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# Association of polymorphic MHC microsatellites with GVHD, survival, and leukemia relapse in unrelated hematopoietic stem cell transplant donor/recipient pairs matched at five HLA loci

**Key words:**
compatibility; graft-*vs*-host disease; hematopoietic stem cell transplantation; human leukocyte antigen; microsatellite markers; polymorphism

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**Abstract:** In order to determine whether matching/mismatching for microsatellite polymorphism provides useful information on acute graft-*vs*-host disease (GVHD), survival, and leukemia relapse in hematopoietic stem cell (HSC) transplantation, we genotyped for polymorphisms at 13 microsatellite loci within the major histocompatibility complex (MHC) of 100 unrelated HSC transplant donor–recipient pairs who were matched at five classical human leukocyte antigen (HLA) loci. A high percentage of allele matching was obtained for five microsatellite loci, DQCARII (96%), MICA (93%), MIB (89%), C1-3-1 (93%), and D6S510 (97%), that are localized within 100 kb of the HLA-DR, HLA-DQ, HLA-B, HLA-C, or HLA-A locus. In contrast, the other eight microsatellites are located farther away from the HLA classical loci and have much lower percentages of allele matching [e.g. tumor necrosis factor a (TNFa) (73%), TNFd (74%), D6S273 (64%), C3-2-11 (46%), C5-3-1 (50%), C5-4-5 (63%), C5-2-7 (68%), and D6S265 (81%)]. Therefore, there were at least eight microsatellite markers with relatively high percentages of mismatches in the donor/recipient pairs with acute or chronic GVHD, poor graft survival, and leukemia relapse. However, there were no statistically significant associations between mismatched donor–recipient pairs at the 13 microsatellite loci and acute or chronic GVHD, graft survival, and leukemia relapse. Nevertheless, allele matching at the microsatellite TNFd locus near the *TNFa* gene was found by the Fisher's exact double-sided test to be significantly associated with decreased survival in the grade III/IV acute GVHD group. Overall, these results suggest that the matching of microsatellite polymorphisms within the HLA region, especially the ones farthest from the classical HLA loci, was not useful indicator for the outcome of HSC transplantation from unrelated donors. In this regard, the future determination of the genome-wide microsatellite genotypes in HLA-matched donor–recipient pairs, outside the MHC, may be a better possibility for identifying minor histocompatibility genes in linkage disequilibria with microsatellites as potential predictive markers for the occurrence of acute GVHD and survival rate in HSC transplantation.

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Hematopoietic stem cell (HSC) transplantation from a human leukocyte antigen (HLA)-matched unrelated donor is an established therapy of hematological malignancies and other hematological or immunological disorders (1–4). The high mortality after unrelated

HSC transplantation is due mostly to severe acute graft-*vs*-host disease (GVHD), and its related complications are still a barrier to the improvement in patient treatment and survival. HLA matching for related or unrelated donors and recipients is considered to be the most important factor influencing the outcome of HSC transplantation. Many investigators have elucidated the clinical significance of HLA factors in unrelated HSC transplantation. Our previous report through the Japan Marrow Donor Program (JMDP) indicated the effect of matching of HLA class I alleles (HLA-A, HLA-B, and HLA-C) on acute GVHD and a possible role for HLA-C in the graft-*vs*-leukemia effect (1, 4). The importance of HLA class II (DR and DQ) matching for GVHD and the association of HLA-C disparity with graft rejection was also reported (5–7). However, even under the conditions of complete HLA allele matching, the risk of acute GVHD and the mortality rate remain high. In fact, GVHD can be a major complication of HSC transplantation even when donor and recipient are siblings and share identical major histocompatibility antigens (8–13). The incidences of grade III and IV and grade II–IV acute GVHD are 18.4 and 35%, respectively, from the data of unrelated HSC transplantation carried out through the National Marrow Donor Program in USA (NMDP) and JMDP, in which patients received transplanted marrow from unrelated donors (14, 15). Mismatching of minor histocompatibility antigens is the most plausible explanation for this high incidence of GVHD. Some minor histocompatibility antigens were identified mainly through the identification and characterization of molecules recognized by T-cell clones that were isolated from the patients who had developed GVHD after HSC transplantation (8–14). Other studies have demonstrated that disparities in minor histocompatibility antigens influence the incidence and severity of acute GVHD (3, 11–15).

The HLA region on chromosome 6p21.3 is one of the most densely gene-packed segments of the human genome and contains, besides the antigen-presenting HLA class I and class II molecules, more than 100 other expressed genes which spread across the entire class II, class III, and class I segments (16, 17). The *HLA* genes, especially classical ones (*HLA-A, -B, -C, -DR, -DQ*, and *-DP*), represent one of the most polymorphic loci on the human genome. Microsatellites defined by 2–6 nucleotide repeats are characterized by a high degree of genetic polymorphism in their repeat number, and they serve as informative polymorphic markers in genetic analyses on gene mapping, haplotype, and linkage disequilibrium (18). More than 250 polymorphic microsatellites were identified and characterized in the entire HLA region (19–22). In Dr Inoko's laboratory, microsatellites in the HLA region have been successfully applied to association mapping of several HLA-associated diseases, such as psoriasis vulgaris (23), Behcet's syndrome (24), rheumatoid arthritis (25), diffuse panbronchio litis (26), azoospermia (27), and non-melanoma skin cancer (28). These microsatellites could also be employed in the identification of genes for minor histocompatibility antigens by similar genome-wide association mapping, based on their tight linkage disequilibria to neighboring genes. In fact, microsatellites in the HLA region represent a valuable source of markers with potential applications in the selection of compatible donor–recipient pairs for bone marrow transplantation, especially in unrelated donors and recipients (29). For example, the microsatellite D6STNFa locus within the HLA class III region was found to correlate well with cytotoxic T-lymphocyte precursor (CTLp) frequency in unrelated bone marrow donors and recipients (30).

In this article, as the first step to conduct genome-wide mapping of minor histocompatibility antigen genes with microsatellites, 13 microsatellite loci within the human MHC were evaluated as potential polymorphic markers for the detection of mismatches within 100 unrelated HSC transplant donor–recipient pairs who were completely matched at five classical HLA loci. The polymorphic microsatellite markers in this group of unrelated donor–recipient pairs were also investigated for their association with GVHD, overall survival, and leukemia relapse, in order to resolve the possibility that some unknown genes within the HLA region may play an important role in HSC transplantation as a minor (or major) histocompatibility antigen.

## Materials and methods

### Subjects

A total of 100 unrelated HSC-transplanted donor–recipient pairs who were treated through the JMDP (1, 4) and who were completely allele matched for the *HLA-A, -B, -C, -DRB1*, and *-DQB1* genes at the high-resolution level were enrolled for this pilot study. All 100 recipients underwent transplantation between 1995 and 2000 for hematopoietic malignancy, with 39 acute myeloblastic leukemia, 27 acute lymphoblastic leukemia, and 34 chronic myeloid leukemia. They survived at least for 3 months after transplantation. The 100 recipients included 57 males and 43 females. The median age of recipients was 28.4 and ranged from 1 to 50 years. The donors included 66 males and 34 females. The median age of donors was 34.6 and ranged from 21 to 51 years. The sex combinations were recipient/donor M/M 42, M/F 16, F/F 18, and F/M 24. Genomic DNAs isolated from all individuals were previously subjected to high-resolution DNA typing for the HLA-A, -B, -C, -DRB1, and -DQB1 loci (1, 4). All 100 recipients received non-T-cell-depleted marrow grafts with immunosuppressive regimes, a combination of cyclosporine and methotrexate for the prophylaxis of GVHD. All the donors and recipients gave informed consent for the study, as approved by the local ethical committee.

### Evaluation of GVHD and relapse of leukemia

The occurrence of acute GVHD was graded according to the established clinical and histopathological criteria (2, 4). The grades were assigned as grade 0 (none), grade I (mild), grade II (modest), grade III (severe), or grade IV (severe), according to the severity of GVHD in the skin, liver, and gastrointestinal tract. The occurrence of chronic GVHD was evaluated in patients who survived more than 100 days after transplantation according to the same criteria that we used before (4). Leukemia relapse/progression was defined as the first hematological evidence of more than 5% of leukemic blasts in the marrow after transplantation or after first remission (1, 4, 15).

### Microsatellite markers within the HLA region and their genotyping

The MHC genomic map of the locations of the 13 microsatellite markers used in this study is shown in Fig. 1. The location of the microsatellites (DQCARII, D6S273, TNFd, TNFa, MICA, MIB, C1-3-1, C3-2-11, C5-3-1, C5-4-5, C5-2-7, D6S265, and D6S510) ranged from DQCARII 5 kb centromeric of the DQA1 loci to D6S510 25 kb centromeric of the HLA-A loci. Polymerase chain reaction (PCR) primer sequences for these markers were previously described (18–21).

Genomic DNA was extracted from peripheral blood of each individual using the Qiagen kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Unilateral primers were labeled with the fluorescence reagent, HEX, 6-FAM, or TET. The reaction mixture was denatured for 3 min at 96°C followed by 35 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 2 min. On PCR completion, about 1 μl (×10 diluted) of PCR product was mixed with 11 μl of formamide and 0.5 μl of internal size standard GS-500 ROX (Applied Biosystems, Foster City, CA). After denaturation for 5 min at 98°C, the mixture was electrophoresed through polymer-bearing DNA-sequencing capillary in an ABI PRISM310 Genetic Analyzer (Applied Biosystems). GENE SCAN software (Applied Biosystems) automatically assigned the fragment sizes. The lengths (bp) of the microsatellite PCR products were determined after collecting all sample data. The accuracy of allele size was attained within 0.5 nucleotide by comparing to known lengths of control DNA obtained from COX or PGF cell lines whose MHC genomic regions have been sequenced (http://www.sanger.ac.uk/HGP/Chr6/MHC/). The length variation between different alleles was >1.5 nucleotides, thus avoiding ambiguities and allowing for a precise definition of the length variation of microsatellite alleles.

### Definition of HLA mismatching

For each donor–recipient pair, we determined whether the recipient allele was the mismatched one that was not shared by the donor (defined as a GVHD direction) or whether the donor allele was the mismatched one that was not shared by the recipient (defined as a rejection direction). In the analysis of factors contributing to acute GVHD, mismatching was investigated in the GVHD direction. In the analysis of factors contributing to relapse or death, mismatching was investigated in both GVHD and rejection directions.

### Statistical analysis

Frequency of microsatellite alleles was calculated from the numbers of genotype counted directly in the sample set. The strength of association was expressed by relative risk (RR), which was calculated from the 2×2 contingency tables. Statistical significance was examined by the Fisher's double-sided exact test. The $P_c$ value (corrected $P$-value) was corrected by multiplying the $P$-value with the number of alleles. The $P_c$ values of less than 0.05 was accepted as statistical significance (Bonferroni's correction).

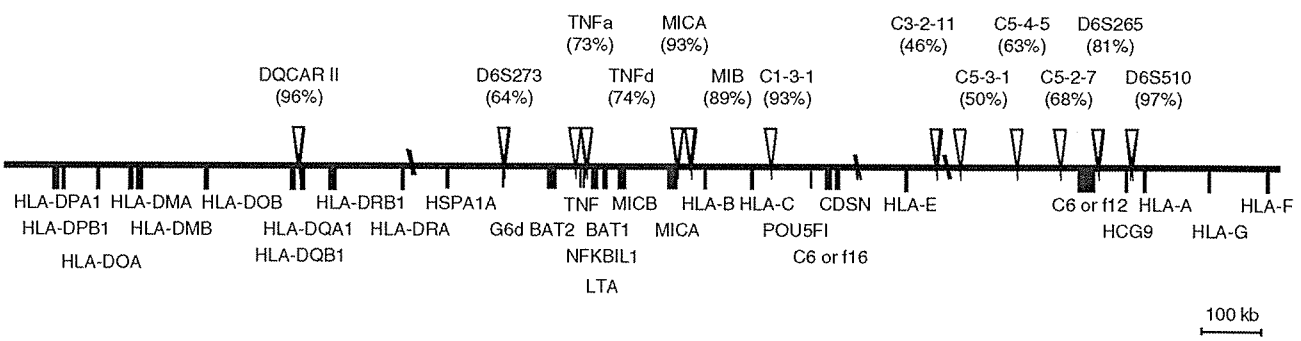


*Fig. 1.* **Physical map of the human leukocyte antigen (HLA) region spanning 3.6 Mb and the location of microsatellite markers used in this study.** Positions of the expressed genes including HLA-A, -B, -C, -DR, and -DQ are indicated along with the positions of microsatellites. Figures in parentheses indicate percentage of allele matching of microsatellites between 100 HLA-identical donors and recipients in hematopoietic stem cell transplantation. ▽, microsatellite markers used in this study; ▮, expressed genes in the HLA region; TNF, tumor necrosis factor.

## Results

### Allele matching–mismatching between 100 unrelated HLA-identical donors and recipients at 13 polymorphic microsatellite markers

Polymorphic genotypes at 13 MHC microsatellite loci were defined for 100 unrelated HLA-A-, HLA-B-, HLA-C-, HLA-DR-, and HLA-DQ-matched donor–recipient pairs in HSC transplantation. As shown in Fig. 1, a high percentage of microsatellite allele matching to HLA alleles was obtained for five microsatellite loci, DQCARII (96%), MICA (93%), MIB (89%), C1-3-1 (93%), and D6S510 (97%), that are localized within 100 kb of the HLA-DR, -DQ, -B, -C, or -A locus. In comparison, the percentage of microsatellite allele matching to HLA alleles was much lower for the other eight microsatellites, TNFd (74%), TNFa (73%), D6S273 (64%), C3-2-11 (46%), C5-3-1 (50%), C5-4-5 (63%), C5-2-7 (68%), and D6S265 (81%), that are localized by more than 100 kb from any of the classical HLA loci.

### Association between microsatellite alleles matched in donor–recipient pairs and different grades of acute GVHD

The numbers and percentages of microsatellite alleles that were matched in donor–recipient pairs with different grades of acute GVHD are summarized in Table 1. Twenty-five of the 100 recipients had not developed acute GVHD (AG = 0), whereas 25 recipients developed grade I acute GVHD, 25 recipients developed grade II, 17 recipients developed grade III, and eight recipients developed grade IV. As summarized in Table 1, none of 13 microsatellites revealed any significant differences in the level of microsatellite allele matching or mismatching among grade 0–IV acute GVHD.

A role of microsatellite mismatching in the development of acute GVHD was further assessed by comparison of the ratio of matching and mismatching between the grade 0 and I and grade II–IV acute GVHD groups in the unrelated donor–recipient pairs. As summarized in Table 2, there was no significant difference ($P > 0.05$) between matched and mismatched donor/recipient pairs for microsatellite loci in acute GVHD grade II–IV and GVHD grade 0 and I. Mismatching at the microsatellite locus D6S265 revealed a possible trend with a significance level of 0.053, which could be followed-up in future studies using much larger numbers of donor/recipient pairs and a greater statistical power.

### Association between microsatellite alleles matched in donor–recipient pairs and chronic GVHD

Information on the incidence of chronic GVHD was obtained from 83 donor–recipient pairs but not from the other 17 pairs due to the death of recipients soon after HSC transplantation. Therefore, there was no information on the incidence of chronic GVHD for five of 25 pairs in the grade II acute GVHD group and 11 of 25 pairs in the grade III and

**Microsatellite allele matching in different grades of acute graft-*vs*-host disease (AG)**

| | Grade (AG) ($n = 25$) | | | |
|---|---|---|---|---|
| Markers | 0 | I | II | III and IV |
| DQCARII | 24 (96%) | 24 (96%) | 23 (92%) | 25 (100%) |
| D6S273 | 14 (56%) | 18 (72%) | 16 (64%) | 16 (64%) |
| TNFd | 18 (72%) | 21 (84%) | 19 (76%) | 16 (64%) |
| TNFa | 17 (68%) | 19 (76%) | 18 (72%) | 19 (76%) |
| MICA | 23 (92%) | 24 (96%) | 24 (96%) | 22 (88%) |
| MIB | 22 (88%) | 23 (92%) | 21 (84%) | 23 (92%) |
| C1-3-1 | 24 (96%) | 23 (92%) | 23 (92%) | 23 (92%) |
| C3-2-11 | 14 (56%) | 11 (44%) | 8 (32%) | 13 (52%) |
| C5-3-1 | 16 (64%) | 14 (56%) | 11 (44%) | 13 (52%) |
| C5-4-5 | 19 (76%) | 16 (64%) | 14 (56%) | 14 (56%) |
| C5-2-7 | 17 (68%) | 17 (68%) | 16 (64%) | 18 (72%) |
| D6S265 | 21 (84%) | 23 (92%) | 18 (72%) | 19 (76%) |
| D6S510 | 23 (92%) | 24 (96%) | 25 (100%) | 25 (100%) |

TNF, tumor necrosis factor.

*Table 1*

**Comparison of the ratio of mismatching and matching in microsatellites between the grade II–IV and grade 0 and I groups**

| | Acute graft-*vs*-host disease | | | | | |
|---|---|---|---|---|---|---|
| | Grade II–IV | | Grade 0 and grade I | | | |
| Markers | Mismatching | Matching | Mismatching | Matching | Relative risk | *P*-value |
| DQCARII | 2 | 48 | 1 | 48 | 2 | 1.0 |
| D6S273 | 14 | 32 | 15 | 32 | 0.93 | 1.0 |
| TNFd | 9 | 35 | 9 | 39 | 1.11 | 1.0 |
| TNFa | 9 | 37 | 13 | 36 | 0.67 | 0.472 |
| MICA | 4 | 46 | 3 | 47 | 1.36 | 1.0 |
| MIB | 6 | 44 | 5 | 45 | 1.23 | 1.0 |
| C1-3-1 | 4 | 46 | 3 | 47 | 1.36 | 1.0 |
| C3-2-11 | 26 | 21 | 24 | 25 | 1.29 | 0.548 |
| C5-3-1 | 19 | 24 | 17 | 30 | 1.4 | 0.520 |
| C5-4-5 | 11 | 28 | 13 | 35 | 1.06 | 1.0 |
| C5-2-7 | 13 | 34 | 13 | 34 | 1 | 1.0 |
| D6S265 | 12 | 37 | 4 | 44 | 3.57 | 0.053 |
| D6S510 | 0 | 49 | 2 | 47 | 0 | 0.495 |
| SEX | 21 | 29 | 19 | 31 | 1.18 | 0.838 |

TNF, tumor necrosis factor.

*Table 2*

grade IV group. In addition, there was no significant ($P > 0.05$) association of mismatching at any of 13 microsatellites loci with the incidence of chronic GVHD before or after stratification of the different grades of acute GVHD (data not shown).

### Association of microsatellite allele matching or mismatching with survival

In this data set, there were 59 recipients who had survived and 41 who had died since transplantation. A univariate analysis revealed no significant correlation between microsatellite matching and survival. Correlations between microsatellite matching and survival were then performed separately within groups of patients having the same grade of acute GVHD. Table 3 summarizes the effect of microsatellite allele matching or mismatching on overall survival in the grade 0, I, II, III, or IV acute GVHD group in which the survival rates were 22 of 25, 20 of 25, 12 of 25, and 5 of 25, respectively. As expected, the more moderate acute GVHD increases the chance of survival, because the high mortality rate after HSC transplantation was mainly due to severe acute GVHD (RR = 10.19, $P < 0.05$). Matching at the microsatellite TNFd locus was associated with decreased survival in the grade III/IV acute GVHD group (survival rate, 20% in matched and 75% in mismatched recipient) with a weak significance ($P = 0.040$ by the two-sided Fisher's exact test) that disappeared after the Bonferroni's correction. Matching at all other microsatellite loci was not significant.

### Association between microsatellite alleles matched in donor–recipient pairs and relapse of leukemia

The effect of microsatellite allele mismatching on relapse of leukemia in the grade 0, I, II, III, or IV acute GVHD group was examined. There was no significant ($P > 0.05$) association of mismatching at any of 13 microsatellites loci with the incidence of relapse of leukemia (data not shown).

## Discussion

The microsatellite markers that were used in this study can be divided simply into two groups, those with a high percentage (88–97%) of allele matching (strong match) and those with a relatively lower percentage (46–81%) of matching (weak match) to HLA alleles. The alleles of the five microsatellite loci that matched strongly to HLA alleles were within 100 kb of the HLA alleles HLA-DR, -DQ, -B, -C, and -A, and they were DQCARII (96%), MICA (93%), MIB (89%), C1-3-1 (93%), and D6S510 (97%). Of this group, the MIB microsatellite locus that is located between the *MICA* and *HLA-B* genes had the lowest allelic association (89%) with the HLA-B alleles

**Association of matching of microsatellite markers with overall survival stratified by acute graft-*vs*-host disease grades**

| | Grade (AG) ($n = 25$) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | I | | II | | III and IV | |
| Markers | Alive = 22 | Dead = 3 | Alive = 20 | Dead = 5 | Alive = 12 | Dead = 13 | Alive = 5 | Dead = 20 |
| DQCARII | 21 (95%) | 3 (100%) | 19 (95%) | 5 (100%) | 11 (92%) | 12 (92%) | 5 (100%) | 20 (100%) |
| D6S273 | 12 (55%) | 2 (67%) | 14 (70%) | 4 (80%) | 6 (50%) | 10 (77%) | 2 (40%) | 14 (70%) |
| TNFd#[a] | 15 (68%) | 3 (100%) | 18 (90%) | 3 (60%) | 9 (60%) | 10 (77%) | 1 (20%) | 15 (75%) |
| TNFa | 16 (73%) | 1 (33%) | 15 (75%) | 4 (80%) | 9 (60%) | 9 (69%) | 4 (80%) | 15 (75%) |
| MICA | 20 (91%) | 3 (100%) | 19 (100%) | 5 (100%) | 11 (92%) | 12 (92%) | 5 (100%) | 17 (85%) |
| MIB | 19 (86%) | 3 (100%) | 18 (90%) | 5 (100%) | 11 (92%) | 10 (77%) | 5 (100%) | 18 (90%) |
| C1-2-5 | 17 (77%) | 2 (67%) | 9 (45%) | 4 (80%) | 11 (92%) | 9 (69%) | 4 (80%) | 14 (70%) |
| C1-3-1 | 22 (100%) | 2 (67%) | 18 (90%) | 5 (100%) | 12 (100%) | 12 (92%) | 5 (100%) | 18 (90%) |
| C3-2-11 | 12 (55%) | 2 (67%) | 8 (40%) | 3 (60%) | 2 (17%) | 6 (46%) | 3 (60%) | 10 (50%) |
| C5-3-1 | 9 (41%) | 1 (33%) | 8 (40%) | 2 (40%) | 6 (50%) | 6 (46%) | 4 (80%) | 8 (40%) |
| C5-4-5 | 5 (23%) | 1 (33%) | 4 (20%) | 2 (40%) | 6 (50%) | 5 (38%) | 2 (40%) | 9 (45%) |
| C5-2-7 | 6 (27%) | 1 (33%) | 5 (25%) | 3 (60%) | 5 (42%) | 4 (31%) | 0 | 1 (5%) |
| D6S265 | 4 (18%) | 0 | 1 (5%) | 1 (20%) | 4 (33%) | 3 (23%) | 1 (20%) | 5 (25%) |
| D6S510 | 2 (9%) | 0 | 1 (5%) | 0 | 0 | 0 | 0 | 0 |

TNF, tumor necrosis factor.
[a]Grade III and IV: $P < 0.040$; relative risk = 12.00.

*Table 3*

in a genomic region that is known to have relatively high-sequence diversity between different haplotypes (31). On the other hand, the eight microsatellite loci that had relatively weaker allelic matches with the *HLA* genes were positioned more than a 100 kb away from the HLA loci. This finding clearly shows that microsatellites are in strong linkage disequilibria with the nearby HLA loci when the distance from microsatellites to the HLA loci is less than 100 kb. This confirms that the distance between microsatellites and the nearest HLA loci in linkage disequilibrium is similar to the distance between microsatellites and disease-susceptible loci in linkage disequilibrium that was determined in our previous association studies of several HLA-associated diseases, using microsatellites in the HLA region (23–28). Therefore, our proposal to detect minor histocompatibility antigen genes by genome-wide mapping with 30,000 polymorphic microsatellites corresponding to one every 100 kb on the human genome is highly feasible, although the major and minor histocompatibility antigen genes may be different from each other in the strength, transmission, and evolutionary history of genetic trait. Furthermore, the five microsatellites, DQCARII, MICA, MIB, C1-3-1, and D6S510, that have a strong allelic association with classical HLA class I and class II genes may have a complementary role in determining HLA identity between donors and recipients in transplantation, especially in unrelated donor–recipient pairs, as previously suggested (29, 32).

Potential roles of microsatellite alleles as genetic markers in the development of acute and chronic GVHDs, the survival rate, and relapse of leukemia in HSC transplantation were also investigated. The population studied here was restricted to the patients who received unmanipulated marrow grafts, and the donor–recipient pairs were selected non-randomly toward allele-matched cases for HLA-A, -B, -C, -DR, and -DQ. There is a variety of risk factors that may effect the selected population, including histoincompatibility, age, sex mismatch, prophylaxis for acute GVHD (33), the intensity of the conditioning regimen, and the use of certain pre- and post-transplantation immunosuppressive agents. These confounding risk factors did not appear to introduce any bias into the analysis ($P = 0.68$) of the present study. In general, none of 13 microsatellites revealed any difference in the level of microsatellite allele matching or mismatching among the grade 0–IV acute GVHD. The only novel finding of the present study was that matching at the microsatellite TNFd locus was associated with decreased survival in the grade III/IV acute GVHD group but with relatively weak significance ($P = 0.040$). This result might show some negative effect of microsatellite matching to survival, which is independent of the GVHD status. In a previous study of 11 MHC microsatellite markers, only the microsatellite TNFa locus was significantly associated with CTLp frequency in unrelated bone marrow donor–recipient pairs (30). It is notable that TNFd is localized in the vicinity of the *TNFA* gene that encodes a ligand for NK receptors and is deeply involved in transplantation immunity in terms of control of cytokine production (34). The small sample size for our finding on the TNFd microsatellite locus (only 3 of 25 patients with grade 0 acute GVHD died, and 5 of 25 grade III/IV were alive) is a limiting factor that prevents further statistical interpretation. More definite conclusion should await larger cohort studies.

There is a school of thought that matching for non-HLA sequences in the MHC, in addition to the HLA loci, is important in transplantation for matching unrelated recipient–donor pairs as a complete haplotype, as this would be equivalent to providing an HLA-identical sibling donor (35, 36). Matching for TNF microsatellites may lower CTLp frequencies (30), and block matching of non-HLA sequences 30 kb centromeric of HLA-B may favor less severe GVHD (37). However, in our study, the 13 MHC microsatellite markers revealed no significant correlations with acute or chronic GVHD, survival, or leukemia relapse in 100 HSC recipients who were matched with their unrelated donors at five HLA loci. This suggests that matching for non-HLA sequences, especially outside the HLA gene clusters or blocks, may not be so important for reducing the rates and severity of GVHD, survival, or leukemia relapse, and that it may be better to look for minor histocompatibility gene effects outside the MHC. In this regard, we now propose to identify minor histocompatibility antigen genes by genome-wide mapping with 30,000 polymorphic microsatellites that correspond to one microsatellite every 100 kb on the human genome. In order to conduct this genome-wide microsatellite-based association study, it will be necessary to recruit a much larger number of donor–recipient pairs than we have in this study to efficiently identify potential predictive markers for the occurrence of and severity of acute and chronic GVHDs, survival, and leukemia relapse in unrelated HLA-identical HSC transplantation.

## References

1. Sasazuki T, Juji T, Morishima Y et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. *N Engl J Med* 1998: **339**: 1177–85.
2. Kernan NA, Bartsch G, Ash RC et al. Analysis of 462 transplantations from unrelated donors facilitated by the National Marrow Donor Program. *N Engl J Med* 1993: **328**: 593–602.
3. Hansen JA, Gooley TA, Martin PJ et al. Bone marrow transplants from unrelated donors for patients with chronic myeloid leukemia. *N Engl J Med* 1998: **338**: 962–8.

4. Morishima Y, Sasazuki T, Inoko H et al. The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. *Blood* 2002: **99**: 4200–6.
5. Petersdorf EW, Longton GM, Anasetti C et al. The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, and DR identical unrelated donor marrow transplantation. *Blood* 1995: **86**: 1606–13.
6. Petersdorf EW, Longton GM, Anasetti C et al. Association of HLA-C disparity with graft failure after marrow transplantation from unrelated donors. *Blood* 1997: **89**: 1818–23.
7. Petersdorf EW, Gooley TA, Anasetti C et al. Optimizing outcome after unrelated marrow transplantation by comprehensive matching of HLA class I and II alleles in the donor and recipient. *Blood* 1998: **92**: 3515–20.
8. Grumet FC, Hiraki DD, Brown BWM et al. CD31 mismatching affects marrow transplantation outcome. *Biol Blood Marrow Transplant* 2001: **7**: 503–12.
9. Ishikawa Y, Kashiwase K, Okai M et al. Polymorphisms in the coding region of mtDNA and effects on clinical outcome of unrelated bone marrow transplantation. *Bone Marrow Transplant* 2001: **28**: 603–7.
10. Juji T, Watanabe Y, Ishikawa Y et al. Human platelet alloantigen (HPA)-5a/b mismatch decreases disease-free survival in unrelated bone marrow transplantation. *Tissue Antigens* 1999: **54**: 229–34.
11. Goulmy E, Schipper R, Pool J et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* 1996: **334**: 281–5.
12. Tseng LH, Lin MT, Hansen JA et al. Correlation between disparity for the minor histocompatibility antigen HA-1 and the development of acute graft-versus-host disease after allogeneic marrow transplantation. *Blood* 1999: **15**: 2911–4.
13. Gallardo D, Arostegui JI, Balas A et al. Disparity for the minor histocompatibility antigen HA-1 is associated with increased risk of acute graft-versus-host disease (GvHD) but it does not affect chronic GvHD incidence, disease-free survival or overall survival after allogeneic human leucocyte antigen-identical sibling donor transplantation. *Br J Haematol* 2001: **114**: 931–6.
14. Akatsuka Y, Nishida T, Kondo E et al. Identification of a polymorphic gene, BCL2A1, encoding two novel hematopoietic lineage-specific minor histocompatibility antigens. *J Exp Med* 2003: **197**: 1489–500.
15. Kodera Y, Morishima Y, Kato S et al. Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. *Bone Marrow Transplant* 1999: **24**: 995–1003.
16. Shiina T, Tamiya G, Oka A et al. Molecular dynamics of MHC genesis unraveled by sequence analysis of the 1,796,938-bp HLA class I region. *Proc Natl Acad Sci USA* 1999: **96**: 13282–7.
17. Shiina T, Tamiya G, Oka A, Takishima N, Inoko H. Genome sequencing analysis of the 1.8 Mb entire human MHC class I region. *Immunol Rev* 1999: **167**: 193–9.
18. Foissac A, Salhi M, Cambon-Thomsen A. Microsatellites in the HLA region: 1999 update. *Tissue Antigens* 2000: **55**: 477–509.
19. Tamiya G, Ota M, Katsuyama Y et al. Twenty-six new polymorphic microsatellite markers around the HLA-B, -C and -E loci in the human MHC class I region. *Tissue Antigens* 1998: **51**: 337–46.
20. Tamiya G, Shiina T, Oka A et al. New polymorphic microsatellite markers in the human MHC class I region. *Tissue Antigens* 1999: **54**: 221–8.
21. Matsuzaka Y, Makino S, Inoko H et al. New polymorphic microsatellite markers in the human MHC class III region. *Tissue Antigens* 2001: **57**: 397–404.
22. Cullen M, Malasky M, Harding A, Carrington M. High-density map of short tandem repeats across the human major histocompatibility complex. *Immunogenetics* 2003: **54**: 900–10.
23. Oka A, Tamiya G, Inoko H et al. Association analysis using refined microsatellite markers localizes a susceptibility locus for psoriasis vulgaris within a 111 kb segment telomeric to the ***HLA-C*** gene. *Hum Mol Genet* 1999: **8**: 2165–70.
24. Mizuki N, Ota M, Yabuki K et al. Localization of the pathogenic gene of Behcet's disease by microsatellite analysis of three different populations. *Invest Ophthalmol Vis Sci* 2000: **41**: 3702–8.
25. Ota M, Katsuyama Y, Kimura A et al. A second susceptibility gene for developing rheumatoid arthritis in the human MHC is localized within a 70-kb interval telomeric of the ***TNF*** genes in the HLA class III region. *Genomics* 2001: **71**: 263–70.
26. Keicho N, Ohashi J, Tamiya G et al. Fine localization of a major disease-susceptibility locus for diffuse panbronchiolitis. *Am J Hum Genet* 2000: **66**: 501–7.
27. Matsuzaka Y, Makino S, Okamoto K et al. Susceptibility locus for non-obstructive azoospermia is localized within the HLA-DR/DQ subregion: primary role of DQB1*0604. *Tissue Antigens* 2002: **60**: 396–9.
28. Oka A, Hayashi H, Tomizawa M et al. Localization of a non-melanoma skin cancer susceptibility region within the major histocompatibility complex by association analysis using microsatellite markers. *Tissue Antigens* 2003: **61**: 203–10.
29. Carrington M, Wade J. Selection of transplant donors based on MHC microsatellite data. *Hum Immunol* 1996: **50**: 151–4.
30. Moghaddam PH, Zwinderman A, Kazemi M et al. D6STNFa microsatellite locus correlates with CTLp frequency in unrelated bone marrow donor–recipient pairs. *Hum Immunol* 1998: **59**: 295–301.
31. Gaudieri S, Kulski JK, Dawkins RL, Gojobori T. Extensive nucleotide variability within a 370 kb sequence from the central region of the major histocompatibility complex. *Gene* 1999: **238**: 157–61.
32. Foissac A, Fort M, Clayton J et al. Microsatellites in the HLA region: HLA prediction and strategies for bone marrow donor registries. *Transplant Proc* 2001: **33**: 491–2.
33. Ratanatharathorn V, Ayash L, Lazarius HM et al. Chronic graft-versus-host disease: clinical manifestation and therapy. *Bone Marrow Transplant* 2001: **28**: 121–9.
34. Schmaltz C, Alpdogan O, Muriglan SJ et al. Donor T cell-derived TNF is required for graft-versus-host disease and graft-versus-tumor activity after bone marrow transplantation. *Blood* 2003: **101**: 2440–5.
35. Christiansen FT, Witt CS, Dawkins RL. Questions in marrow matching: the implications of ancestral haplotypes for routine practice. *Bone Marrow Transplant* 1991: **8**: 83–6.
36. Witt C, Moghaddam HP, van der Meer R et al. Matching for TNF microsatellites is strongly associated with matching for other non-HLA MHC sequences in unrelated bone marrow donor–recipient pairs. *Hum Immunol* 1999: **60**: 862–6.
37. Tay GK, Witt CS, Christiansen FT, Charron D et al. Matching for MHC haplotypes results in improved survival following unrelated bone marrow transplantation. *Bone Marrow Transplant* 1995: **15**: 381–5.

# Unrelated bone marrow transplantation for non-Hodgkin lymphoma: a study from the Japan Marrow Donor Program

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**There is little information available regarding the outcome of unrelated bone marrow transplantation (BMT) for non-Hodgkin lymphoma (NHL). Therefore, we retrospectively analyzed the data of 124 patients who underwent unrelated BMT through the Japan Marrow Donor Program (JMDP) between July 1992 and August 2001. The overall survival (OS), progression-free survival (PFS), cumulative incidences of disease progression, and nonprogression mortality at 3 years after BMT were 49.7%, 42.6%, 24.5%, and 32.9%, respectively, with a median follow-up duration of 565 days among survivors. The incidence of grades II-IV acute graft-versus-host disease (GVHD) was 40.9%. Recipient age, previous history of autologous transplantation, and chemosensitivity at transplantation were independent prognostic factors for OS and PFS. The development of grades II-IV acute GVHD was associated with lower incidence of disease progression after transplantation, which suggested the existence of a graft versus lymphoma effect. Unrelated BMT should be considered as a treatment option for patients with high-risk NHL without an HLA-matched related donor. (Blood. 2004;103:1955-1960)**



## Introduction

Hematopoietic stem cell transplantation for non-Hodgkin lymphoma (NHL) has been mainly performed using an autologous graft, because the incidence of treatment-related mortality after allogeneic transplantation is as high as 57%.[1] However, relapse is a frequent cause of treatment failure after autologous transplantation.[2,3] The lower relapse rate after allogeneic transplantation and the recent development of supportive treatments to decrease the risk of treatment-related mortality have facilitated the use of allogeneic transplantation for NHL.[1] However, an HLA-matched sibling is available for less than half of the patients. Transplantation from an unrelated donor is a possible alternative for patients who do not have a suitable related donor. To date, however, little information is available regarding the outcome of allogeneic transplantation from an unrelated donor for NHL. Therefore, we retrospectively analyzed the outcome of unrelated bone marrow transplantation for NHL using the database of the Japan Marrow Donor Program (JMDP). The purpose of this study was to elucidate the feasibility of unrelated bone marrow transplantation for NHL and to evaluate the impact of a potential graft-versus-lymphoma effect.

## Patients and methods

### Patients and transplantation procedure

From July 1992 to August 2001, 124 patients with non-Hodgkin lymphoma (NHL) underwent bone marrow transplantation from a serologically HLA-A, -B, and -DR matched unrelated donor identified through the Japan Marrow Donor Program (JMDP). The application of unrelated transplantation was decided at each center. Fourteen of 19 patients who underwent unrelated transplantation in the first complete remission (CR1) had high-grade lymphoma.

Transplantation was performed according to the protocol of each center, and therefore the conditioning regimen and graft-versus-host disease (GVHD) prophylaxis varied among patients (Table 1). However, 90% of the patients received a total body irradiation (TBI)–containing conditioning regimen. Prophylaxis against GVHD was performed with cyclosporine A or tacrolimus combined with methotrexate with or without corticosteroid in all but one patient. At transplantation, 60 patients were in complete remission (CR) and 60 were not (non-CR). Among the 43 patients whose CR status was reported in detail, 19, 18, 5, and 1 were in CR1, CR2, CR3, and CR4, respectively. Seventy-six patients had chemosensitive disease at transplantation, whereas 33 patients had chemoresistant disease. In this study we defined patients who achieved CR or partial remission (PR) before transplantation as chemosensitive, and patients with responses less than PR were defined as chemoresistant in the same way as previous studies.[4,5] Before unrelated donor transplantation, 18 of 101 patients had undergone high-dose therapy and autologous stem cell transplantation (HDT/ASCT). Information regarding previous treatments except for HDT/ASCT or the results of genomic typing were not available in the dataset.

### Histology

The JMDP requested the histologic subtype of NHL according to a unique classification system that was a slight modification of the Working Formulation.[6] However, the respective centers used different classification systems, such as the Working Formulation, Kiel,[7] Lymphoma Study Group

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A complete list of the centers in Japan that participated in the bone marrow transplantations for non-Hodgkin lymphoma facilitated by the Japan Marrow Donor Program (JMDP) appears in the "Appendix."

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**Table 1. Patient characteristics**

| Characteristic | Value |
|---|---|
| Sex, n, M/F | 78/46 |
| Median age at transplantation, y (range) | 29 (1-59) |
| Median interval from diagnosis to transplantation, d (range) | 470 (183-2329) |
| **Histology, n** | |
| Low-grade | 10 |
| Follicular lymphoma | 9 |
| Small lymphocytic lymphoma | 1 |
| Intermediate-grade | 42 |
| Peripheral T-cell lymphoma, unspecified | 14 |
| NK-cell lymphoma | 12 |
| Anaplastic large cell lymphoma | 6 |
| Diffuse large B-cell lymphoma | 5 |
| Angioimmunoblastic lymphoma | 1 |
| Mantle cell lymphoma | 1 |
| High-grade | 60 |
| Lymphoblastic lymphoma | 39 |
| Adult T-cell leukemia/lymphoma | 15 |
| Burkitt lymphoma | 5 |
| Unclassified | 12 |
| **Previous history of HDT/ASCT, n** | |
| Yes | 18 |
| No | 83 |
| ND | 23 |
| **Disease status at transplantation, n** | |
| CR | 60 (CR1 19, CR2 18, CR3 5, CR4 1) |
| Non-CR | 60 |
| ND | 4 |
| **Chemosensitivity at transplantation, n** | |
| Sensitive | 76 |
| Resistant | 33 |
| ND | 15 |
| **Conditioning regimen, n** | |
| TBI-containing regimen | 111 |
| Non-TBI regimen | 13 |
| **GVHD prophylaxis, n** | |
| CsA ± MTX ± steroid | 76 |
| TCR ± MTX ± steroid | 44 |
| CsA + TCR ± MTX ± steroid | 3 |
| MTX alone | 1 |

HDT/ASCT indicates high-dose therapy and autologous stem cell transplantation; CR, complete remission (CR1, CR2, CR3, CR4; the first, second, third, and fourth CR, respectively); ND, not described; TBI, total body irradiation; GVHD, graft-versus-host disease; CsA, cyclosporine A; MTX, methotrexate; TCR, tacrolimus.

(LSG),[8] Revised European-American Classification of lymphoid neoplasms (REAL),[9] and World Health Organization (WHO) systems.[10] In this study, we grouped the histology into low grade, intermediate grade, and high grade as usually accepted in daily practice. There were 10, 42, and 60 patients with low-, intermediate-, and high-grade lymphoma, respectively. Histologic subtypes in detail are described in Table 1. The histologic subtype or grade was unclassified in 12 cases. Transplantation for lymphoblastic lymphoma (LBL) and adult T-cell leukemia/lymphoma (ATLL) was included as in other studies focusing on allogeneic transplantation for NHL.[11,12]

### Data management and statistical considerations

Data were collected by the JMDP using a standardized report form. Follow-up reports were submitted at 100 days, 1 year, and annually after transplantation. Overall survival (OS) was defined as days from transplantation to death from any cause. Progression-free survival (PFS) was defined as days from transplantation to disease progression or death from any cause. Nonprogression mortality was defined as death without disease progression. Patients who were alive at the last follow-up date were censored. Survival was calculated using the Kaplan-Meier method. To evaluate the influence of confounding factors for survival, the log-rank test was used for univariate analyses and proportional hazard modeling was used for multivariate analyses. Cumulative incidences of acute GVHD and disease progression were calculated using the Gray method,[13] considering death without acute GVHD and death without disease progression as respective competing risks. The effects of acute and chronic GVHD on survival and disease progression were analyzed among patients who survived without disease progression at 60 and 150 days after transplantation, respectively.[14,15] This landmark method was used to exclude bias that may arise from including patients who died too early to develop GVHD in the group without GVHD.

## Results

### Survival and disease progression

Of the 124 patients, 69 were alive with a median follow-up duration of 565 days (range, 82 to 2217 days) after transplantation (Table 2). The overall 3-year OS and PFS were 49.7% and 42.6%, respectively (Figure 1A). Cumulative incidences of disease progression and nonprogression mortality at 3 years were 24.5% and 32.9%, respectively (Figure 1B). Disease progression was observed in 26 patients, and the median time from transplantation to disease progression was 109 days (range, 0 to 1079 days). Notably, only 1 patient developed disease progression more than 500 days after

**Table 2. Transplantation outcome**

| | Value |
|---|---|
| **Alive/dead, n** | 69/55 |
| Median follow-up for survivors, d (range) | 565 (82-2217) |
| **Cause of death** | |
| Progression, n | 17 |
| Median days after transplantation (range) | 165 (2-1106) |
| Death without progression, n | 36 |
| Median days after transplantation (range) | 72 (8-718) |
| GVHD, n | 10 |
| Infection, n | 9 |
| IP, n | 6 |
| VOD, n | 3 |
| Renal failure, n | 2 |
| ARDS, n | 2 |
| Others: pericarditis, hemorrhage, cerebral infarction, RRT, n | 4 |
| Not described, n | 2 |
| **Disease progression, n** | 26 |
| Median days after transplantation (range) | 109 (0-1079) |
| **Engraftment, n** | |
| Engraftment | 115 |
| Rejection | 2 |
| Death within 20 days | 7 |
| **Acute GVHD, n*** | |
| Grade 0 | 31 |
| Grade I | 37 |
| Grade II | 30 |
| Grade III | 7 |
| Grade IV | 10 |
| **Chronic GVHD, n†** | |
| None | 47 |
| Limited | 17 |
| Extensive | 24 |
| Not described | 5 |

GVHD indicates graft-versus-host disease; IP, interstitial pneumonitis; VOD, venoocclusive disease; ARDS, acute respiratory distress syndrome; RRT, regimen-related toxicity.

*Acute GVHD was evaluated among patients who achieved engraftment and survived more than 20 days after transplantation.

†Chronic GVHD was evaluated among patients who survived more than 100 days after transplantation.

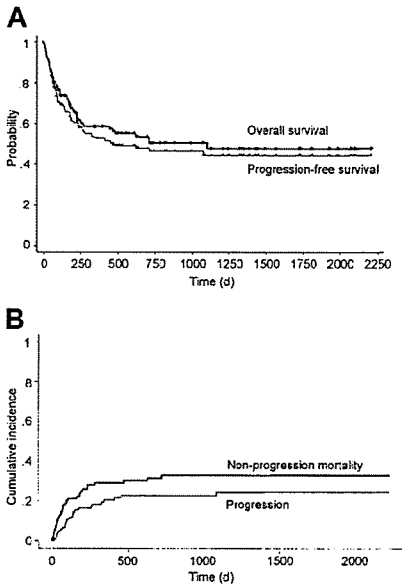


**Figure 1. Survival, progression, and nonprogression mortality after transplantation.** Overall survival, progression-free survival (A), and cumulative incidences of disease progression and nonprogression mortality (B) after unrelated bone marrow transplantation for non-Hodgkin lymphoma.

transplantation. The cause of death was related to disease progression in 17, whereas 36 died without disease progression (Table 2). The major cause of transplantation-related death within 100 days after transplantation was GVHD in 8, infection in 5, venoocclusive disease in 3, acute respiratory distress syndrome in 2, interstitial pneumonitis in 2, renal failure in 2, and other causes in 2.

### Engraftment and GVHD

Seven patients died within 20 days after transplantation, and therefore engraftment could not be evaluated. In the others, 2 rejected the graft and 115 achieved engraftment. Among the latter 115 patients, 47 developed grades II-IV acute GVHD (Table 2) with a cumulative incidence of 47.5% (Figure 2). Seven and 10 patients experienced grade III and IV acute GVHD, respectively. Among the 93 patients who were alive at 100 days after transplantation, 17 and 24 developed limited and extensive chronic GVHD, respectively.

### Influence of pretransplantation factors

We evaluated the effects of pretransplantation factors on OS after transplantation and identified 3 independent significant risk factors: chemosensitivity before transplantation (chemosensitive versus chemoresistant, relative risk 0.28, 95% confidence interval [CI] 0.15-0.52, $P < .0001$); previous history of HDT/ASCT (yes versus no, relative risk 0.40, 95% CI 0.20-0.79, $P = .0087$); and patient age (less than 40 years versus 40 years or more, relative risk 0.42, 95% CI 0.22-0.81, $P = .0092$) at transplantation (Tables 3 and 4; Figure 3A-C). These 3 factors were also identified as independent risk factors for PFS (data not shown), probably because only a few patients survived after disease progression and the OS and PFS curves were almost superimposed. We further analyzed the impact of disease status among patients who had chemosensitive disease at bone marrow transplantation; 19 were in first CR, 24 were in a later CR, and 16 were in PR. However, there was no significant difference in OS or PFS among them (data not shown). Among 13 deaths in patients with previous history of HDT/ASCT, 11 were from transplantation-related causes before day 100 (median, 56; range, 13 to 97 days after transplantation). Two patients died from

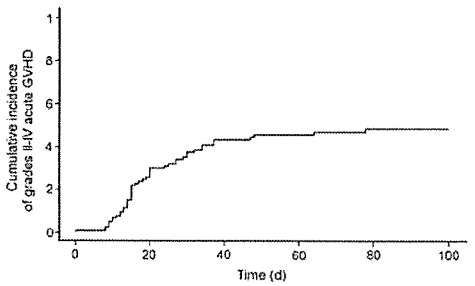


**Figure 2. Cumulative incidence of grades II-IV acute graft-versus-host disease.**

disease progression on day 48 and 458, respectively. However, 5 of 6 patients who survived more than 100 days after transplantation were progression free at a median follow-up of 1339 days (range, 493 to 2217 days) after transplantation. Furthermore, we evaluated the impact of histologic grade on outcome. However, there was no significant difference in OS, PFS, or cumulative incidence of disease progression among these histologic grades (Figure 3D and data not shown).

### Influence of acute and chronic GVHD

We analyzed the relationship between the development of acute GVHD and the transplantation outcome. In this study, all but 2 patients developed acute GVHD before day 60. Thus, we defined day 60 as a landmark for this analysis. The cumulative incidence of disease progression at 3 years after transplantation was significantly lower in patients who developed grades II-IV acute GVHD (5.9% versus 33.2%, $P = .0053$; Figure 4B). This effect was preserved even when it was adjusted for the chemosensitivity before transplantation using proportional hazard modeling (relative risk 0.15, 95% CI 0.03-0.65, $P = .012$). This inverse correlation between the development of acute GVHD and disease progression

**Table 3. Prognostic factors in univariate analyses**

| | 3-year OS, % | P |
|---|---|---|
| **Sex** | | .70 |
| Male | 47.7 | |
| Female | 53.1 | |
| **Age at transplantation** | | .0036 |
| Less than 40 y | 57.1 | |
| 40 y or more | 28.7 | |
| **Histology** | | .80 |
| Low grade | 60.0 | |
| Intermediate grade | 53.2 | |
| High grade | 47.6 | |
| **Previous HDT/ASCT** | | .0006 |
| Yes | 27.8 | |
| No | 56.3 | |
| **Chemosensitivity** | | < .0001 |
| Chemosensitive | 63.2 | |
| Chemoresistant | 22.8 | |
| **Disease status** | | .0011 |
| CR | 61.4 | |
| Non-CR | 32.0 | |
| **Preparative regimen** | | .29 |
| TBI-containing | 50.8 | |
| Non-TBI | 40.0 | |
| **Days from diagnosis to transplantation** | | .53 |
| Less than 365 d | 44.8 | |
| 365 d or more | 52.1 | |

OS indicates overall survival; HDT/ASCT, high-dose therapy and autologous stem cell transplantation; CR, complete remission; TBI, total body irradiation.

**Table 4. Prognostic factors in multivariate analysis**

| | Relative risk | 95% CI | P |
|---|---|---|---|
| Age less than 40 y | 0.42 | 0.22-0.81 | .0092 |
| No previous HDT/ASCT | 0.40 | 0.20-0.79 | .0087 |
| Chemosensitive disease | 0.28 | 0.15-0.52 | < .0001 |

CI indicates confidence interval.

suggested the existence of a graft-versus-lymphoma (GVL) effect. However, there was no significant difference in 3-year OS (61.4% versus 58.8%, $P = .63$; Figure 4A) or PFS (58.9% versus 48.9%, $P = .28$) between patients with and without grades II-IV acute GVHD. When we classified patients into those who developed grades III-IV acute GVHD and those who did not, there was a trend for lower incidence of disease progression ($P = .13$) but worse OS ($P = .075$) in patients with acute GVHD. The influence of chronic GVHD was evaluated similarly, with day 150 after transplantation defined as a landmark. However, the cumulative incidence of disease progression at 3 years after transplantation was not different between those with and without chronic GVHD (12.3% versus 14.5%, $P = .80$).

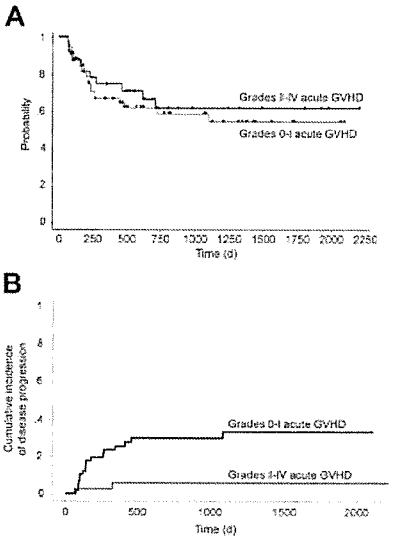


**Figure 4. Survival and progression according to development of acute GVHD.** OS (A) and cumulative incidence of disease progression (B) grouped by the development of grades II-IV acute graft-versus-host disease among patients who were alive without disease progression at 60 days after transplantation.

### Results in specific histologic subtypes

Kaplan-Meier estimates of OS of patients with peripheral T-cell lymphoma (n = 14), natural killer (NK)–cell lymphoma (n = 12), LBL (n = 39), and ATLL (n = 15), which were the 4 major histologic subtypes in this study, are shown in Figure 5. OS of patients with peripheral T-cell lymphoma, which is considered to be associated with poor prognosis,[16-18] appeared to be favorable after unrelated allogeneic transplantation with several long-term survivors (3-year OS, 75.0%), although this study contained only a small number of patients. In contrast, the result for ATLL was poor, and there were no survivors beyond 500 days after transplantation.

## Discussion

In this study, we analyzed the outcome of bone marrow transplantation from an unrelated donor for NHL performed through the JMDP. In a similar study from the National Marrow Donor Program (NMDP),[19] both OS and PFS were estimated to be 30% at 2 years. The outcome in the present study appeared to be more favorable than that in the NMDP study, which could be attributed to the lower incidence of grades III-IV acute GVHD in the present study (15% versus 30%). This observation is compatible with previous studies showing lower incidence of acute GVHD among Japanese than among whites, which might reflect less diverse genetic background in Japan.[20,21]

The association between the development of GVHD and reduced disease progression rate has been inconsistent among previous studies.[4,11,22] In this study, the development of grades II-IV acute GVHD was associated with a lower incidence of disease progression after transplantation. This result supports the existence of a potential GVL effect. Because the previous studies nearly exclusively included transplantation from an

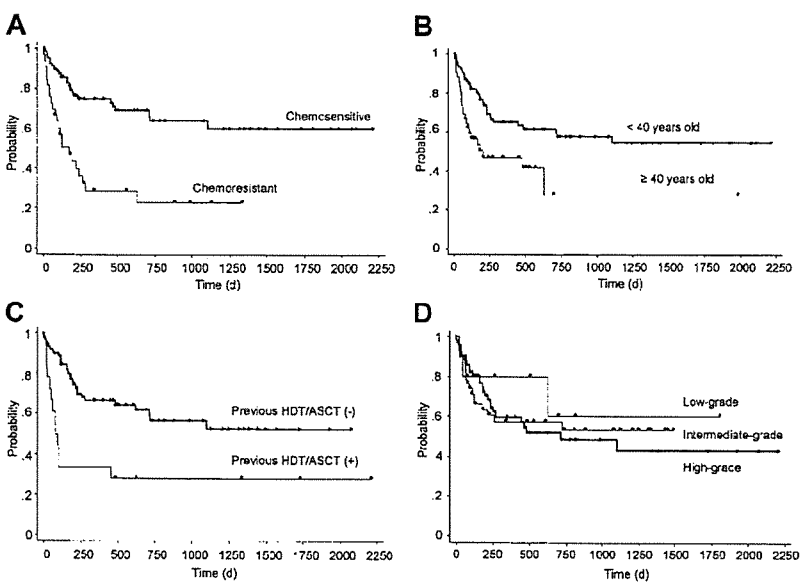


**Figure 3. Overall survival according to pretransplantation factors.** OS grouped by chemosensitivity at transplantation (A), age (B), previous history of autologous transplantation (HDT/ASCT) (C), and histologic grade (D).

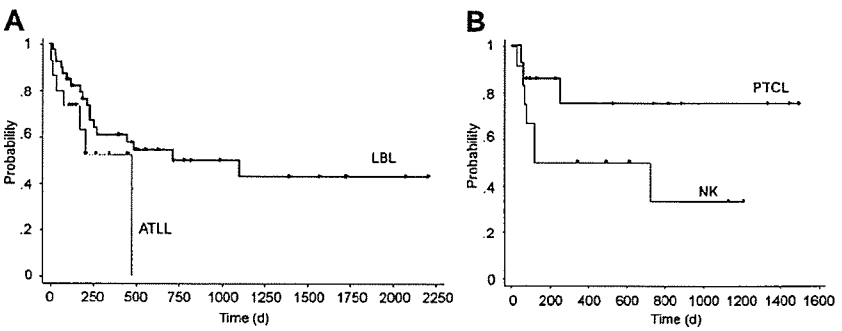


**Figure 5. Overall survival of specific histologic subtypes.** (A) Adult T-cell leukemia/lymphoma (ATLL) and lymphoblastic lymphoma (LBL); (B) peripheral T-cell lymphoma (PTCL) and NK-cell lymphoma (NK).

HLA-matched sibling, the use of unrelated donor might have facilitated the GVL effect. Nevertheless, there was no difference in OS or PFS between patients with and without grades II-IV acute GVHD, because the decreased incidence of disease progression was counterbalanced by the increased incidence of transplantation-related mortality. An association between the development of chronic GVHD and the incidence of disease progression was not observed. We suppose that the main reason we failed to observe this association is insufficient statistical power due to the paucity of disease progression (only 9 patients) beyond day 150, a landmark for the analysis.

Chemosensitivity at transplantation was identified as a major prognostic factor for OS. Patients with chemosensitive disease at transplantation were associated with lower nonprogression mortality, a lower incidence of disease progression, and better survival. These results may raise the question whether the effect of allogeneic transplantation was from the GVL effect or from high-dose therapy before transplantation. However, this study strongly suggested the existence of the GVL effect, because the incidence of disease progression was significantly lower in patients who developed acute GVHD, even after adjusted for chemosensitivity before transplantation. Previous history of HDT/ASCT was also a strong prognostic factor. Because the median survival for patients with NHL who had a relapse after HDT/ASCT is extremely short (less than 12 months),[23] allogeneic transplantation using conventional or reduced-intensity conditioning is being evaluated in this population. In this study, 11 patients (61%) with a previous history of HDT/ASCT died within 100 days after transplantation from transplantation-related causes. On the other hand, most of the patients who survived beyond day 100 were progression free with long follow-up, suggesting a benefit of allogeneic transplantation to suppress disease progression. Therefore, strategies to decrease transplantation-related mortality are important, especially for patients after HDT/ASCT failure. Allogeneic transplantation with reduced-intensity conditioning is an option that deserves further evaluation.[24]

LBL, ATLL, peripheral T-cell lymphoma, and NK-cell lymphoma were the 4 major histologic subtypes in this population. The composition of the histologic subtypes in this study was different from that of NHL in general and that in previous studies focusing on allogeneic transplantation for NHL.[4,12] Higher ratios of peripheral T-cell lymphoma, NK-cell lymphoma, and ATLL would, at least in part, be a reflection of the histologic population of NHL in Japan.[25] Because the long-term results with conventional therapy and/or HDT/ASCT for ATLL had been always dismal,[26] allogeneic transplantation even for patients in CR is being tested in a clinical trial in several centers in Japan.[27] The long-term results for peripheral T-cell lymphoma and NK-cell lymphoma with conventional therapy, especially in patients with advanced or relapsed disease, were also poor.[16-18,28] The high ratio of LBL might be a reflection of Japanese physicians' preference to perform allogeneic transplantation for LBL in CR1.

Transplantation outcome in each histologic subtype should be evaluated further to select patients who will benefit from unrelated transplantation. In this study, there was no difference in OS or PFS among the 3 grades. Although no patient with low-grade lymphoma had disease progression at a median follow-up of 513 days, the number of patients was too small and follow-up period was too short to draw a definite conclusion. Based on the results of this study, unrelated donor bone marrow transplantation deserves to be evaluated in patients with peripheral T-cell lymphoma and NK-cell lymphoma, considering the poor results after conventional chemotherapy for these subtypes.[16-18,28] Finally, although the outcome of patients with ATLL was poor in this study, this treatment strategy should not be abandoned because both the number of the patients and the follow-up duration were not enough.

In conclusion, allogeneic bone marrow transplantation from an unrelated donor appeared to be a feasible treatment option for patients with high-risk NHL. Further study is required to determine detailed indications for unrelated transplantation for NHL, including histologic subtype and disease status.

## Appendix

The following centers in Japan participated in the bone marrow transplantations for NHL facilitated by the JMDP: Hokkaido University Hospital, Sapporo Hokuyu Hospital, Japanese Red Cross Asahikawa Hospital, Iwate Medical University Hospital, Tohoku University Hospital, Yamagata University Hospital, National Cancer Center Central Hospital, Tokyo Metropolitan Komagome Hospital, Nihon University Itabashi Hospital, Jikei University Hospital, Keio University Hospital, University of Tokyo Hospital, National Tokyo Medical Center, Kanagawa Children's Medical Center, Kanagawa Cancer Center, Tokai University Hospital, Chiba University Hospital, Saitama Cancer Center Hospital, Saitama Medical School Hospital, Jichi Medical School Hospital, Saiseikai Maebashi Hospital, Gunma University Hospital, Niigata Cancer Center Hospital, Saku Central Hospital, Japanese Red Cross Nagoya First Hospital, Nagoya Daini Red Cross Hospital, Meitetsu Hospital, Nagoya University Hospital, Aichi Cancer Center, Showa Hospital, Kanazawa University Hospital, Kinki University Hospital, Osaka University Hospital, Osaka Medical Center and Research Institute for Maternal and Child Health, Matsushita Memorial Hospital, Kansai Medical University Hospital, Hyogo College of Medicine Hospital, Hyogo Medical Center for Adults, Kyoto University Hospital, Tottori University Hospital, Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital, Ehime Prefectural Central Hospital, National Okayama Medical Center, Kyushu University Hospital, Harasanshin General Hospital, Hamanomachi General Hospital, National Kyushu Cancer Center, St Mary's Hospital, Saga Prefectural Hospital, Nagasaki University Hospital, Kumamoto National Hospital, and Oita Medical University Hospital.

## References


1. Bierman PJ. Allogeneic bone marrow transplantation for lymphoma. Blood Rev. 2000;14:1-13.
2. Philip T, Guglielmi C, Hagenbeek A, et al. Autologous bone marrow transplantation as compared with salvage chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. N Engl J Med. 1995;333:1540-1545.
3. Horning SJ, Negrin RS, Hoppe RT, et al. High-dose therapy and autologous bone marrow transplantation for follicular lymphoma in first complete or partial remission: results of a phase II clinical trial. Blood. 2001;97:404-409.
4. Dhedin N, Giraudier S, Gaulard P, et al. Allogeneic bone marrow transplantation in aggressive non-Hodgkin's lymphoma (excluding Burkitt and lymphoblastic lymphoma): a series of 73 patients from the SFGM database. Societe Francaise de Greffe de Moelle. Br J Haematol. 1999;107:154-161.
5. van Besien K, Sobocinski KA, Rowlings PA, et al. Allogeneic bone marrow transplantation for low-grade lymphoma. Blood. 1998;92:1832-1836.
6. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. The Non-Hodgkin's Lymphoma Pathologic Classification Project. Cancer. 1982;49:2112-2135.
7. Lennert K, Fellar A. Histopathology of Non-Hodgkin's Lymphomas. 2nd ed. New York, NY: Springer-Verlag; 1992.
8. Suchi T, Tajima K, Nanba K, et al. Some problems on the histopathological diagnosis of non-Hodgkin's malignant lymphoma — a proposal of a new type. Acta Pathol Jpn. 1979;29:755-776.
9. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood. 1994;84:1361-1392.
10. Jaffe E, Harris NL, Stein H, Vardiman JW. Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001.
11. Chopra R, Goldstone AH, Pearce R, et al. Autologous versus allogeneic bone marrow transplantation for non-Hodgkin's lymphoma: a case-controlled analysis of the European Bone Marrow Transplant Group Registry data. J Clin Oncol. 1992;10:1690-1695.
12. van Besien KW, Mehra RC, Giralt SA, et al. Allogeneic bone marrow transplantation for poor-prognosis lymphoma: response, toxicity and survival depend on disease histology. Am J Med. 1996;100:299-307.
13. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999;18:695-706.
14. Anderson JR, Cain KC, Gelber RD. Analysis of survival by tumor response. J Clin Oncol. 1983;1:710-719.
15. Sullivan KM, Weiden PL, Storb R, et al. Influence of acute and chronic graft-versus-host disease on relapse and survival after bone marrow transplantation from HLA-identical siblings as treatment of acute and chronic leukemia. Blood. 1989;73:1720-1728.
16. Rudiger T, Weisenburger DD, Anderson JR, et al. Peripheral T-cell lymphoma (excluding anaplastic large-cell lymphoma): results from the Non-Hodgkin's Lymphoma Classification Project. Ann Oncol. 2002;13:140-149.
17. Gisselbrecht C, Gaulard P, Lepage E, et al. Prognostic significance of T-cell phenotype in aggressive non-Hodgkin's lymphomas. Groupe d'Etudes des Lymphomes de l'Adulte (GELA). Blood. 1998;92:76-82.
18. Melnyk A, Rodriguez A, Pugh WC, Cabannillas F. Evaluation of the Revised European-American Lymphoma classification confirms the clinical relevance of immunophenotype in 560 cases of aggressive non-Hodgkin's lymphoma. Blood. 1997;89:4514-4520.
19. Bierman PJ, Molina L, Nelson G, King R, Fay J, Champlin R. Matched unrelated donor (MUD) allogeneic bone marrow transplantation for Non-Hodgkin's lymphoma (NHL): results from the National Marrow Donor Program (NMDP) [abstract]. Proc Am Soc Clin Oncol. 1999;18:3a.
20. Morishima Y, Kodera Y, Hirabayashi N, et al. Low incidence of acute GVHD in patients transplanted with marrow from HLA-A, B, DR-compatible unrelated donors among Japanese. Bone Marrow Transplant. 1995;15:235-239.
21. Kodera Y, Morishima Y, Kato S, et al. Analysis of 500 bone marrow transplants from unrelated donors (UR-BMT) facilitated by the Japan Marrow Donor Program: confirmation of UR-BMT as a standard therapy for patients with leukemia and aplastic anemia. Bone Marrow Transplant. 1999;24:995-1003.
22. Ratanatharathorn V, Uberti J, Karanes C, et al. Prospective comparative trial of autologous versus allogeneic bone marrow transplantation in patients with non-Hodgkin's lymphoma. Blood. 1994;84:1050-1055.
23. Vose JM, Bierman PJ, Anderson JR, et al. Progressive disease after high-dose therapy and autologous transplantation for lymphoid malignancy: clinical course and patient follow-up. Blood. 1992;80:2142-2148.
24. Khouri IF, Saliba RM, Giralt SA, et al. Nonablative allogeneic hematopoietic transplantation as adoptive immunotherapy for indolent lymphoma: low incidence of toxicity, acute graft-versus-host disease, and treatment-related mortality. Blood. 2001;98:3595-3599.
25. The World Health Organization classification of malignant lymphomas in Japan: incidence of recently recognized entities. Lymphoma Study Group of Japanese Pathologists. Pathol Int. 2000;50:696-702.
26. Tsukasaki K, Maeda T, Arimura K, et al. Poor outcome of autologous stem cell transplantation for adult T cell leukemia/lymphoma: a case report and review of the literature. Bone Marrow Transplant. 1999;23:87-89.
27. Kami M, Hamaki T, Miyakoshi S, et al. Allogeneic haematopoietic stem cell transplantation for the treatment of adult T-cell leukaemia/lymphoma. Br J Haematol. 2003;120:304-309.
28. Kwong YL, Chan AC, Liang R, et al. CD56+ NK lymphomas: clinicopathological features and prognosis. Br J Haematol. 1997;97:821-829.

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BRIEF COMMUNICATION

# Analysis of Chromosomal Imbalances in de novo CD5-Positive Diffuse Large-B-cell Lymphoma Detected by Comparative Genomic Hybridization

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We recently demonstrated that the prognosis for de novo CD5-positive ($CD5^+$) diffuse large-B-cell lymphoma (DLBCL) is markedly worse than that for CD5-negative ($CD5^-$) DLBCL. Our findings also suggested that on the basis of its clinical features $CD5^+$ DLBCL may constitute a unique disease category. However, the genetic basis for these two categories has not been established. Therefore, we performed comparative genomic hybridization analysis (CGH) of 26 cases of $CD5^+$ DLBCL and 44 cases of $CD5^-$ DLBCL. Several identical changes in $CD5^+$ and $CD5^-$ DLBCLs were found, such as gains of 3q, 9p, 12q, 13q, and 18q and losses of 1p, 6q, 17p, and 19p. However, distinct differences between the two categories were also detected. These included gains of 11q21-q24 ($P = 0.032$) and 16p ($P = 0.005$) in $CD5^+$ DLBCL, and loss of 16p ($P = 0.028$) in $CD5^-$ DLBCL. A comparison with results reported for mantle cell lymphoma, chronic lymphocytic leukemia, and Richter's syndrome demonstrated that the CGH pattern of $CD5^+$ DLBCL was markedly different. This indicates that $CD5^+$ DLBCL constitutes a disease category distinct from that of $CD5^-$ DLBCL and other $CD5^+$ malignancies. 

Diffuse large-B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma and is characterized by aggressive behavior (Gatter and Warnke., 2001) and a heterogeneous clinical course. DLBCL is heterogeneous at the genetic level (Harris et al., 1994), but the genetic basis of this heterogeneity is poorly understood. Translocation of the *BCL6* gene has been identified in 10%–20% of DLBCLs (Chaganti et al., 1998; Cigudosa et al., 1999), but the clinical significance of the translocation remains unclear.

DLBCL expresses various pan-B surface markers, such as CD19, CD20, CD22, and CD79a, and 10% express the CD5 antigen (Matolocsy et al., 1995). We previously reported that $CD5^+$ DLBCL patients had a survival rate significantly inferior to those with $CD5^-$ DLBCL (Harada et al., 1999; Yamaguchi et al., 2002). This finding suggested that $CD5^+$ DLBCL might constitute a clinically distinct subgroup of DLBCLs. Therefore, in this study, we investigated the genomic alterations in $CD5^+$ and $CD5^-$ DLBCLs, detected by comparative genomic hybridization (CGH) analysis.

We selected DNA samples from nine patients with de novo $CD5^+$ DLBCL at the Aichi Cancer Center, from eight at the Fujita Health University School of Medicine, and from nine at the Mie University School of Medicine, as well as from 40 patients with $CD5^-$ DLBCL at the Aichi Cancer Center and from four at Mie University School of Medicine. All patients were selected from the previously reported DLBCL patients whose DNA was available (Harada et al., 1999; Yamaguchi et al., 2002; Table 1). The results of the CGH analysis for $CD5^+$ and $CD5^-$ DLBCL are shown in Figures 1a and 1b, respectively.

We identified several common and distinct regions of genomic alterations in the two groups. Gains of 11q21-q24 and 16p were characteristic of $CD5^+$ DLBCL. In $CD5^+$ DLBCL, 5 of 26 cases showed gains at 16p (19%) and 7 of 26 cases at 11q21-q24 (26%). These genetic alterations did not occur simultaneously except in two cases. It there-

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TABLE 1A. Regions of Gains and Losses in 26 Cases of CD5$^+$ DLBCL Determined by Comparative Genomic Hybridization Analysis

| Case no. | Sex | Age | Gains | Losses |
|---|---|---|---|---|
| 1 | M | 61 | 1p36-pter, 2p21-pter, 4p13-pter, 6p21.3-pter, 8p, 12q24.2-pter, 16p, 17pter-q11.2, 19 | 1q21.1-q31, 2p13-p16, 7p13-pter, 7q21.2-q22, 11q14.3-qter, 13q14.3-qter, 15q15-qter, 17q22-qter, 20p, 21q, Xp11, Xq22, Xq26 |
| 2 | F | 71 | 1p33-pter, 9q34.1-qter, 10p11.2-pter, 14q32-qter, 17pter-q11.2 | 3p14.1-qter, 7p12-pter, 11q22.1-qter, 13q14.3-qter, 16p11.2-pter, 16q, Xp11.4-p21, Xq25-q26 |
| 3 | F | 36 | 1p32-pter, 12q24-qter, 16p13-pter, 19 | 3q, 11, Xp11, Xq26 |
| 4 | M | 51 | 19p | 3, 11q22.1-qter, 18 |
| 5 | M | 67 | 17 | 3qcen-qter, 11q14.3-q23.1, 18 |
| 6 | F | 72 | 14q32-qter | 12pter-q12, 12q21.3-qter, 16p, 22q11.2-qter, Xp11, Xq25 |
| 7 | M | 58 | | 6p22.3-pter, 16p11.2-pter |
| 8 | F | 41 | 1p36-pter | 3p14.3-pter, 3q13.2-qter, 12pcen-p12.1, 12q15-q23, 16p12-p13.3, 18, Xp11, Xq25 |
| 9 | F | 38 | | 12q21.3-q23, Xp11.4-p21 |
| 10 | F | 61 | 1p33-pter, 17p, 19p | 13q21.3-qter, Xq26 |
| 11 | F | 76 | 6q16.2-qter, 8p21.1-pter | 8q21.1-qter, 18q11.2-qter, Xq25-q27 |
| 12 | F | 82 | 19p | 13q21.3-qter, Xp11, Xq25 |
| 13 | F | 76 | 19p | 8q23-q24.2, 18q21.2-q22, Xp11.4-p21, Xq25 |
| 14 | F | 71 | 9q13-q31 | 3p14.1-pter, 3q13.3-qter, 10, Xq25-q26 |
| 15 | M | 74 | 17 | 8q22.3-qter |
| 16 | M | 59 | 1p34-pter, 6q22-qter, 14q32-qter, 17p | 11q22.1-qter, 13q21-qter, 18 |
| 17 | F | 62 | 1q41-qter, 2q33-q37, 17p | 5ptel-q14, 9p, 11q14-qter, Xp11.4-p21.1 |
| 18 | F | 68 | 1p34-pter, 8p12-pter, | 6p21.3-pter, 9p23-pter, 9q22.3-q33, 10p, 13q21.3-qter, Xp11, Xq21 |
| 19 | F | 64 | 6q21-q25, 17p | 1q22-qter, 4q21.3-qter, 7, 10ptel-q21, 12pcen-p12, 12qcen-q24, 19q13.3-qter, Xp11.4-p21.1, Xq25-q26 |
| 20 | M | 54 | 1p34-pter, 4p15-pter, 5p15-pter, 9q34, 17p-p21, 19p, 21q22, 22 | 1q25-q32, 2q22-q37, 3, 5q14-q23.3, 6p22.3-pter, 13q22.2-q23.3 |
| 21 | F | 59 | 1q41-qter, 13q14.3-q21.1, 17p12-pter | 1q24, 3, 18q, Xp11-p21 |
| 22 | M | 60 | 6q21-qter | 2, 11q14.3-qter, 16p12-pter, 16q, |
| 23 | F | 57 | | 9p21-pter, Xp21, Xq25-q26 |
| 24 | M | 39 | 6q25.3-qter | 15q15-q21.1, 18q21-qter, 21q21 |
| 25 | F | 44 | 1p32-pter, 9q34-qter, 12q24-qter, 15qcen-q22.3, 16p13-pter, 17p | 1q21.3-qter, 5ptel-q23, 7q31-q32, Xq26 |
| 26 | F | 73 | 6q15-qter, 17 | 4, 6p, 12p11.1-p12, 14q13-qter, 20p |

fore is conceivable that gains of either 11q21-q24 or 16p (38%) are characteristic of CD5$^+$ DLBCL. We also found that the patients with CD5$^+$ DLBCL featuring 11q amplification had significantly better survival than the other patients with CD5$^+$ DLBCL ($P = 0.02$, data not shown). However, a larger number of patients are needed to confirm this result. Barth et al. (2001) found that gain of 11q21-q24 was preferentially observed in diffuse large-B-cell lymphomas of the gastrointestinal tract without a small-cell component (giDLBL). However, no surface markers such as the CD5 molecule were used, and so the relationship between giDLBL and CD5$^+$ DLBCL has remained unclear. Rao et al. (1998) reported that 16p12 was seen as a region of high amplification in some DLBCL cases and that the gain of 16p12 accounted for less than 10%. These findings may reflect the gains identified in CD5$^+$ DLBCL in our study.

Common regions of gain for CD5$^+$ and CD5$^-$ DLBCLs were 3q, 9p, 12q, 13q, 18q, and the X chromosome (Table 2). These gains were previously reported as the most common gains in DLBCL (Rao et al., 1998; Berglund et al., 2002). However, 3q amplification has also been detected in mantle cell lymphoma (MCL; Monni et al., 1998) and 12q amplification in follicular cell lymphoma (Bentz et al., 1996), indicating that these gains are not associated with any specific disease types.

As for mature B-cell neoplasms, CD5 is expressed in most patients with MCL, chronic lymphocytic leukemia (B-CLL), and diffuse large-B-cell lymphomas transformed from B-CLL

TABLE 1B. Regions of Gains and Losses in 44 Cases of CD5⁻ DLBCL Determined by Comparative Genomic Hybridization Analysis

| Case no | Sex | Age | Gains | Losses |
|---|---|---|---|---|
| 1 | F | 46 | 6q15-qter | 2q22-q37.2, 5p13.3-pter, 6p, Xp11, Xq27 |
| 2 | F | 46 | 6p21.3-p23, 6q21-qter, 8pter-q13, 10, 21q | 1q22-qter, 3q26.1-qter, 6p12, Xp11, Xq27 |
| 3 | M | 38 | 1p32.3-pter, 6q15-qter, 17p, 19 | 3p22-p24.3, 3q13.3-qter, 7q22-qter, 11, 13q21.2-q31, 18q |
| 4 | F | 60 | 1p31.3-pter, 6q15-qter, 9q12-qter, 17, 22q | 1q22-qter, 3q25.3-qter, 4q27-q32, 6p, 9p21-pter, 12q15-q21.3, Xp11.4-p21.1, Xq25-q26 |
| 5 | M | 61 | 1p34.3-pter, 6q14-q22.1 | 3p12-qter, 5q15-q31.1, 6p12-p21.3, 7ptel-q31.3, 18 |
| 6 | F | 65 | 1p32.3-pter, 6q15-qter, 9q12-qter, 16p, 17 | 3p12-qter, 5p13.3-q23.3, 6p, 8q22.3-qter, 12p, 12q15-q22, 13q12.2-qter, Xp11, Xq22 |
| 7 | M | 39 | 6q15-q21 | 3p12-qter, 5, 6p12-p21.3, 7p13-pter, 18q12.2-qter |
| 8 | F | 48 | 6q14-q22.1, 10q11.2-q23.3, 17, 22q | 5, 6p, 7p12-p21, 7q21.1-qter, 12p, 12q15-q23, 13q12.3-qter, 18q21.1-qter, Xp11, Xq22 |
| 9 | M | 58 | 1p32.3-pter, 16p12-pter, 19, 22q | 3p14.3-qter, 5q15-q31.3, 9p21-pter, 18q |
| 10 | F | 61 | 1p34.3-pter, 7p14-pter, 19 | 2p13-pter, 3q26.1-qter, 5, 9p21-pter, 12q11.-q21.1, 18, Xp11.4-p21.1, Xq25 |
| 11 | F | 34 | 17, 19 | 3, 5p13.3-p14, 5q21-qter, 7p12-p21, 7q31.1-qter, 13q12.3-qter, 18q12.2-qter, Xp11.4, Xq25 |
| 12 | M | 47 | 1p, 4pter-q13.1, 16, 17p | 1q21.3-qter, 13q21.3-q31, Xp11.4-p21.1, Xq25-q26 |
| 13 | M | 68 | 17p | 3, 9q21.3-q31, 17q12-q21.3, 18q, Xq25-q26 |
| 14 | M | 33 | | 3q27-qter |
| 15 | F | 46 | 1p32.3-pter, 17p,20q12-qter, 21q | 3q, 7q21.1-qter, 7p13-p15.2, 9p, 9q21.3-q32, 12p, 18, Xp11.4-p21, Xq25-q26 |
| 16 | F | 58 | 16q23-qter, 17p | 8q22.3-qter, 13q14.3-q31, 18, Xp11-p21, Xq23-q24, Xq26-q27 |
| 17 | M | 46 | | 1q31-q41, 2p |
| 18 | M | 54 | 17p, 22q | 3q13.3-qter, 7p12-p21, 7q31.1-qter, 14q23-q24.2, Xp11, Xq22, Xq25 |
| 19 | F | 53 | 9q34.1-qter, 17ptel-q21.3 | 13q21.3-q31, Xp11, Xq25 |
| 20 | M | 60 | 17p | 3q13.3-q21, 18q12.1-q21.1 |
| 21 | M | 56 | | 12p, 12q13.2-q24.1 |
| 22 | M | 52 | | 5ptel-q12, 18q12.1-qter |
| 23 | M | 54 | 1p36-pter, 3p14.1-pter, 9q34, 16, 17, 19p, 22q | 1q23-q31, 2q24-q34, 6p, 11, Xp11.4-p21.1, Xq26 |
| 24 | M | 55 | 1p, 4, 15q22.3-qter, 17p, 19p | 1q22-q31, 2p14-p15, 7p15.1-pter, 13q14.3-qter, 14q21-q22 |
| 25 | M | 70 | 1p34-pter, 17 | 4q23-q26, 9p, 21q, Xp11, Xq22, Xq25-q26 |
| 26 | F | 70 | 9q34, 16p, 17p, 22q | 13q22-q31, Xp11-p21, Xq26-q27 |
| 27 | F | 48 | 1p34.3-pter, 9q34.1-qter, 16p, 17p | 13q21.3-q31, Xp11-p21, Xq25 |
| 28 | F | 63 | 1p34.3-pter, 9p34.1-qter, 17p | 3q13.2-q26.2, 4q22-q26, 12p11, 12q21.3-q22, 13q21.3, Xp11-p21, Xq22, Xq25-q26 |
| 29 | M | 50 | 14q32, 17p | 5q21-q23.1, 9p, 15q22-qter |
| 30 | M | 69 | 6q14-qter, 7q31.1-q31.3 | 6p21.3-pter, 18q12.3-qter |
| 31 | M | 76 | 1p32.1-pter, 9q34.1-qter, 16p13.1-pter, 17p | 6p21.3-pter, 7q21.3-q31.3, Xp11-p21.2, Xq25-q26 |
| 32 | M | 52 | 1p36, 17p | 11p11-p12, Xp11-p21, Xq26 |
| 33 | F | 54 | 1p36, 16p13, 17p13 | 18q, Xp11.4-p21.1, Xq26 |
| 34 | M | 26 | 16p13, 17p | 6p23-pter, 9p21-pter, 11q22-qter, Xq25-q26 |
| 35 | M | 61 | 1p36, 19 | |
| 36 | F | 76 | 1p31.3-pter, 7q31.3-qter, 9q34-qter, 10q26-qter, 15q23-qter, 16, 17, 20q, 22q | 2p15-pter, 3ptel-q26.1, 9p, 11, 12q14-q22, 14q12-q24, 18, Xp11, Xq23-q26 |
| 37 | M | 72 | 6q21-q23 | 18q21.2-qter |
| 38 | F | 45 | 1p31.3-pter, 3p12-p23, 4p14-pter, 10q15-qter, 11p15, 13qcen-q14, 17 | 1q44, 2p13-p15, 5q15, 7p14-pter, 7q31-qter, 9p23-pter, 10q21, 11q14-qter, 18q12-qter |
| 39 | F | 68 | 1p36-pter, 16p13.3-pter, 19 | 9p24-pter, Xp11-p21 |
| 40 | M | 76 | 1p33-pter,12q24-qter, 14q32-qter, 16, 17, 20q | 1q, 3, 11q14.3-q21 |
| 41 | M | 65 | 1p34-pter, 9q32-qter, 16, 17p, 22 | 1q22-q31, Xp11-p21, Xq21-q23 |
| 42 | M | 76 | 1p36-pter, 16p, 17p | 1q23-q31, 12 |
| 43 | F | 37 | 1p33-pter, 3p14.1-p21.1, 6q23-q24, 16p, 17p, 18q22-qter, 20 | 1q, 7q, 12q13.1-q21.3, 21q, Xp11.4-p21, Xq22 |
| 44 | M | 74 | 1p36-pter, 6q23-qter, 9p, 16p13-pter, 17p, Xp22.3-pter | 11, 18, 19q13.3 |

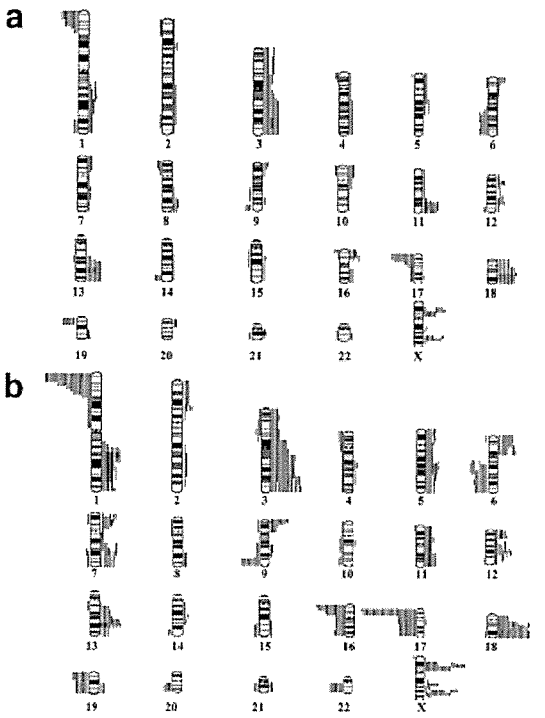

Figure 1. Summary of chromosomal gains and losses in $CD5^+$ and $CD5^-$ DLBCLs detected by CGH: (a) 26 cases of $CD5^+$ DLBCL [under- representation (loss) of DNA is denoted by vertical lines to the left of the ideogram, and overrepresentation (gain) of DNA by vertical lines to the right; bold lines represent high-level gains]; (b) 44 cases of $CD5^-$ DLBCL (as in a).

TABLE 2. Frequent Chromosomal Imbalances Detected in $CD5^+$ and $CD5^-$ DLBCLs

| Chromosomal regions | $CD5^+$DLBCL ($n$ = 26) | $CD5^-$DLBCL ($n$ = 44) | $P$ |
|---|---|---|---|
| **Gains** | | | |
| 3q[a] | 5 (19%) | 14 (31%) | |
| 6p | 4 (15%) | 11 (25%) | |
| 9p23-p24 | 3 (11%) | 10 (22%) | |
| 11q21-q24[a] | 7 (26%) | 3 (6%) | 0.032 |
| 12q[a] | 4 (15%) | 8 (18%) | |
| 13q21-q31 | 7 (26%) | 11 (25%) | |
| 16p | 5 (19%) | 0 | 0.005 |
| 18 | 8 (30%) | 18 (40%) | |
| Xp11-p21 | 12 (46%) | 22 (50%) | |
| Xq25-q26 | 11 (42%) | 18 (40%) | |
| **Losses** | | | |
| 1p | 9 (34%) | 26 (59%) | |
| 6q | 6 (23%) | 12 (27%) | |
| 16p | 3 (11%) | 16 (36%) | 0.028 |
| 17p | 12 (46%) | 30 (68%) | |

[a]Patients with whole-chromosome amplification were not included.

(Richter's syndrome). Monni et al. (1998) reported that a gain of 3q and losses of 8p, 13q14, 11q22, and 6q are frequent aberrations in MCL. In our study, however, such changes were not observed in $CD5^+$ DLBCL except for a gain of 3q. The CGH patterns of MCL and $CD5^+$ DLBCL are thus markedly different. Other studies found that loss of 13q14-q21, 13q32-q34, 9p21, and 11q22-q23 and gain of 3q26-q29, 8q22-q24, and 18q21-q22 are characteristic of B-CLL (Bentz et al., 1995; Karhu et al., 1997). Although gains of 3q26-q29, 8q22-q24, and 18q21-q22 were also observed in our $CD5^+$ DLBCL cases, no losses of 13q, 9p, or 11q22-q24 were found, indicating that the CGH pattern in $CD5^+$ DLBCL is clearly different from that in B-CLL. Matolcsy et al. (1995) reported that DLBCL associated with Richter's syndrome was genotypically different from DLBCL, whereas Beà et al. (2002) found that genomic alteration patterns of B-CLL and Richter's syndrome are very similar. The different CGH patterns of $CD5^+$ DLBCL and B-CLL indicate that the CGH pattern of $CD5^+$ DLBCL is also different from that of Richter's syndrome.

In conclusion, we found distinctly different CGH patterns in $CD5^+$ and $CD5^-$ DLBCLs, confirming that $CD5^+$ DLBCL forms a specific subtype of DLBCL. However, some cases of $CD5^+$ DLBCL did not show these characteristic genomic alterations. This may be explained by the CGH analysis being unable to detect gains or losses smaller than 5–10 Mb (Lichter et al., 2000). Because of this limitation, some samples in our study may have contained undetected regions of minimal genomic alteration, including 11q21-q24 and 16p. A more sensitive method such as matrix (array) CGH analysis therefore may be needed (Pinkel et al., 1998; Wessendorf et al., 2001). Moreover, cDNA microarray expression profiling may help to identify the molecular bases for $CD5^+$ DLBCL. Thus, further studies are needed to examine in greater detail the gene aberrations at 11q21-q24 and 16p for characterization of $CD5^+$ DLBCL.

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## REFERENCES

Barth TFE, Bentz M, Leithuser H, Stilgenbauer S, Siebert R, Schlotter M, Schenk RF, Döhner H, Möller P. 2001. Molecular-

cytogenetic comparison of mucosa-associated marginal zone B-cell lymphoma and large B-cell lymphoma arising in the gastrointestinal tract. Genes Chromosomes Cancer 31:316–325.

Beà S, López Guillermo A, Ribas M, Puig X, Pinyol M, Carrió A, Zamora L, Soler F, Bosch F, Stilgenbauer S, Colomer D, Miró R, Montserrat E, Campo E. 2002. Genetic imbalances in progressed B-cell chronic lymphocytic leukemia and transformed large-cell lymphoma (Richter's syndrome). Am J Pathol 161:957–968.

Bentz M, Huck K, du Manoir S, Joos S, Werner CA, Fischer K, Döhner H, Lichter P. 1995. Comparative genomic hybridization in chronic B-cell leukemias shows a high incidence of chromosomal gains and losses. Blood 85:3610–3618.

Bentz M, Werner CA, Döhner H, Joos S, Barth TF, Siebert R, Schroder M, Stilgenbauer S, Fischer K, Möller P, Lichter P. 1996. High incidence of chromosomal imbalances and gene amplifications in the classical follicular variant of follicle center lymphoma. Blood 88:1437–1444.

Berglund M, Enblad G, Flordal E, Lui W-O, Backlin C, Thunberg U, Sundström C, Roos G, Allander SV, Erlanson M, Rosenquist R, Larsson C, Lagercrantz S. 2002. Chromosomal imbalances in diffuse large B-cell lymphoma detected by comparative genomic hybridization. Mod Pathol 15:807–817.

Chaganti RK, Chen W, Parasa N, Offit K, Louie DC, Dalla-Favera R, Chaganti RSK. 1998. Involvement of BCL6 in chromosomal aberrations affecting band 3q27 in B-cell non-Hodgkin lymphoma. Genes Chromosomes Cancer 23:323–327.

Cigudosa JC, Parsa NZ, Louie DC, Filippa DA, Jhanwar SC, Johansson B, Mitelman F, Chaganti RSK. 1999. Cytogenetic analysis of 363 consecutively ascertained diffuse large B-cell lymphomas. Genes Chromosomes Cancer 25:123–133.

Gatter KC, Warnke RA. 2001. Diffuse large B-cell lymphoma. In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. World health classification of tumors. Pathology & genetics of tumours of haematopoietic and lymphoid tissues. Washington, DC: IARC Press, Lyon. p. 171–174.

Harada S, Suzuki R, Uehira K, Yatabe Y, Kagami Y, Ogura M, Suzuki H, Oyama A, Kodera Y, Ueda R, Morishima Y, Nakamura S, Seto M. 1999. Molecular and immunological dissection of diffuse large B cell lymphoma: CD5+, and CD5 with CD10+ groups may constitute clinically relevant subtypes. Leukemia 13:1441–1447.

Harris NL, Jaffe ES, Stein H. Banks PM, Chan J.K.C, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC, Grogan TM, Issacson PG, Knowlws DM, Mason DY, Muller-Hermelink H-S, Pileri SA, Piris MA, Ralfkiaer E, Warnke RA. 1994. A revised European-American classification of lymphoid neoplasms: A proposal from the International Lymphoma Study Group. Blood 84:1361–1392.

Karhu R, Knuutila S, Kallioniemi O-P, Sanna Siitonen, Aine R, Vilpo L, Vilpo J. 1997. Frequent loss of the 11q14–24 region in chronic lymphocytic leukemia: AQ study by comparative genomic hybridization. Genes Chromosomes Cancer 19:286–290.

Lihter P, Joos S, Bentz M, Lampel S. 2000. Comparative genomic hybridization: Uses and limitations. Semin Haematol 37:348–357.

Matolcsy A, Chadburn A, Knowles DM. 1995. De novo CD5-positive and Richter's syndrome-associated diffuse large B cell lymphomas are genotypically distinct. Am J Pathol 147:207–216.

Monni O, Oinonen R, Eloen E, Franssia K, Terenhovi L, Joensuu H, Knuutia S. 1998. Gain of 3q and deletion of 11q22 are frequent aberrations in mantle cell lymphoma. Genes Chromosomes Cancer 21:298–307.

Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo W, Chen C, Zhai Y, Dairkee SH, Ljung B, Gray JW, Albertson DG. 1998. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nature 20:207–211.

Rao PH, Houldsworth J, Dyomina K, Parsa NZ, Cigudosa JC, Louie DC, Popplewell L, Offit K, Jhanwar SC, Chaganti RSK. 1998. Chromosomal and gene amplification in diffuse large B-cell lymphoma. Blood 92:234–240.

Wessendorf S, Fritz B, Wrobel G, Nessling M, Lampel S, Getdl D, Kepper M, Joos S, Hopman T, Kokocinski F, Döhner H, Bentz M, Schwenen C, Lichter P. 2001. Automated screening for genomic imbalances using matrix-based comparative genomic hybridization. Lab Invest 82:47–60.

Yamaguchi M, Seto M, Okamoto M, Ichinohasama R, Nakamura N, Yoshino T, Suzumiya J, Murase T, Miura I, Akasaka T, Tamaru J, Suzuki R, Kagami Y, Hirano M, Morishima Y, Ueda R, Shiku H, Nakamura S. 2002. De novo CD5+ diffuse large B-cell lymphoma: A clinicopathologic study of 109 patients. Blood 99:815–821.

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# Genome-Wide Array-Based Comparative Genomic Hybridization of Diffuse Large B-Cell Lymphoma: Comparison between CD5-Positive and CD5-Negative Cases

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## ABSTRACT

**Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma and exhibits aggressive and heterogeneous clinical behavior. To genetically characterize DLBCL, we established our own array-based comparative genomic hybridization and analyzed a total of 70 cases [26 CD-positive ($CD5^+$) DLBCL and 44 CD5-negative ($CD5^-$) DLBCL cases]. Regions of genomic aberrations observed in >20% of cases of both the $CD5^+$ and $CD5^-$ groups were gains of 1q21-q31, 1q32, 3p25-q29, 5p13, 6p21-p25, 7p22-q31, 8q24, 11q23-q24, 12q13-q21, 16p13, 18, and X and losses of 1p36, 3p14, 6q14-q25, 6q27, 9p21, and 17p11-p13. Because CD5 expression marks a subgroup with poor prognosis, we subsequently analyzed genomic gains and losses of $CD5^+$ DLBCL compared with those of $CD5^-$. Although both groups showed similar genomic patterns of gains and losses, gains of 10p14-p15 and 19q13 and losses of 1q43-q44 and 8p23 were found to be characteristic of $CD5^+$ DLBCL. By focusing on the gain of 13q21-q34 and loss of 1p34-p36, we were also able to identify prognostically distinct subgroups among $CD5^+$ DLBCL cases. These results suggest that array-based comparative genomic hybridization analysis provides a platform of genomic aberrations of DLBCL both common and specific to clinically distinct subgroups.**

## INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) comprises some 30% of non-Hodgkin's lymphoma cases and is clinically heterogeneous (1, 2). Recent microarray analyses of transcripts of DLBCL specimen have clearly shown biologically distinct subtypes in DLBCL that are also clinically relevant (3, 4). Although several genetic alterations have been identified as etiologically associated with DLBCL (5, 6), genome-wide screening has been insufficient. The recently developed array-based comparative genomic hybridization (array CGH) techniques allow high-throughput analysis of copy number changes of a genome at high resolution and accuracy throughout the whole genome. The quantitative measurement of DNA copy number thus obtained may facilitate identification of tumor-related genes (7–9). We recently established our own array CGH using a glass slide on which 2088 bacterial artificial chromosome/P-1 phage-derived artificial chromosome (BAC/PAC) DNA were spotted in duplicate with an average resolution of 1.5 Mb (10). This enabled us to identify a novel gene, *C13orf25*, as overexpressed in B-cell lymphoma cell lines and DLBCL patients with 13q31-q32 amplifications (10). These results warrant application of array CGH analysis to DLBCL genome widely.

CD5 is a cell surface molecule physiologically expressed on T cells and on subsets of B cells residing in the mantle zone of lymphoid organs and the peritoneal cavity. Clinically, CD5 expression is often associated with chronic lymphocytic leukemia (11) and mantle cell lymphoma (MCL; ref. 12). For DLBCL cases, we have identified CD5 expression as a marker of poor prognosis, whereas CD5-positive ($CD5^+$) DLBCL is associated with elderly onset, female predominance, and the frequent involvement of extranodal sites (13). Microarray analyses of transcripts showing different expressions by $CD5^+$ and CD5-negative ($CD5^-$) DLBCL cases also indicate that $CD5^+$ DLBCL is a distinct disease entity (14, 15).

In this study, we used the array CGH technique to analyze 26 $CD5^+$ and 44 $CD5^-$ DLBCL cases and identified genomic aberrations shared by both groups, as well as those specific to $CD5^+$ DLBCL. Our results may provide both insights into the genomic basis of lymphoma genesis and genetic markers useful for the identification of clinically distinct subgroups of DLBCL.

## MATERIALS AND METHODS

**Patients.** We analyzed DNA samples of 26 cases of *de novo* $CD5^+$ DLBCL and 44 cases of $CD5^-$ DLBCL. These samples were obtained with informed consent from patients at Aichi Cancer Center and collaborating institutions under the approval of the Institutional Review Boards. All patients were reported previously (13, 16, 17). The median age was 61 years and 56 years for the $CD5^+$ and $CD5^-$ cases, respectively. Among the $CD5^+$ cases, 68% were female, 80% were at advanced stages (III to IV), 72% had elevated lactate dehydrogenase, 24% had a poor performance status, and 28% had extranodal site(s) of involvement. In the $CD5^-$ cases, 41% were female, 52% had advanced stages (III to IV), 45% had elevated lactate dehydrogenase, 10% had a poor performance status, and 38% had extranodal site(s) of involvement. All of the samples were obtained from tumors at diagnosis before any treatment was given.

**DNA Samples.** DNA was extracted using a standard phenol chloroform method from lymphoma specimens from a total of 70 DLBCL cases: 26 cases of $CD5^+$ DLBCL and 44 cases of $CD5^-$ DLBCL. Normal DNA was prepared from peripheral-blood lymphocytes of healthy male donors.

**Malignant Lymphoma Cell Line.** Cell line used in this study was OCI-LY13.2 (DLBCL, kindly provided by Dr. Ricardo Dalla-Favera of Columbia University, New York, NY; ref. 18). OCI-LY13.2 was maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% $CO_2$ – 95% air.

**Selection of BAC/PAC Clones (BAC/PACs) for Array CGH.** The array consisted of 2088 BAC/PACs, covering whole human genome with ~1.5 Mb of resolution. BACs were derived from RP11 and RP13 libraries, and PACs were derived from RP1, RP3, RP4, and RP5 libraries. BAC/PACs used were selected based on information from National Center for Biotechnology Information and Ensembl Genome Data Resources.[7,8] These clones were obtained from the BACPAC Resource Center at the Children's Hospital (Oakland

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Research Institute, Oakland, CA).[9] Clones were ordered from chromosomes 1 to 22 and X. Within each chromosome, clones were ordered from chromosomes 1 to 22 and X on the basis of Ensembl Genome Data Resources of Sanger Center Institute, January 2004 version. All of the clones used for array CGH were confirmed for their location on chromosomes by fluorescence *in situ* hybridization. Clone names and their locations on chromosomes are available on request.

**DNA Amplification for Spotting on Slides.** Ten ng of BAC (or PAC) DNA were used as the template for degenerate oligonucleotide-primed PCR (19) with the 5′-amine-modified degenerate oligonucleotide-primed primer (5′-CCGACTCGAGNNNNNNATGTGG-3′ where $n$ = A, C, G, or T) and amplified on a TaKaRa PCR Thermal Cycler MP (TaKaRa, Tokyo, Japan) using Ex Taq polymerase (TaKaRa). A 3-minute, 94°C denaturation step was followed by 25 cycles of 94°C for 30 seconds, a 37°C to 72°C linear ramp for 10 minutes, and 72°C for 1 minute, with a final 7-minute extension at 72°C.

**DNA Spotting and Quality Control of Glass Slides.** Degenerate oligonucleotide-primed PCR products were ethanol precipitated and dissolved in distilled water, and then an equal volume of DNA spotting solution DSP0050 (Matsunami, Osaka, Japan) was added (1 $\mu$g/$\mu$L). The resulting DNA samples were robotically spotted by an inkjet technique (NGK, Nagoya, Japan) in duplicate onto CodeLink-activated slides (Amersham Biosciences, Piscataway, NJ). In this study, we used only glass slides on which it had been confirmed that all 2088 clones had been spotted completely and uniformly in duplicate.

**Array Hybridization.** The array fabrication and hybridization was performed according to the method described by Pinkel *et al.* (20) and Hodgson *et al.* (21). One $\mu$g of tested (tumor or normal) and of referenced (normal) DNA was digested with *Dpn*II and labeled with the BioPrime DNA labeling system (Invitrogen-Life Technologies, Inc., Tokyo, Japan) using Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, Piscataway, NJ) for the tested and referenced DNA, respectively. Unincorporated fluorescent nucleotides were removed by means of Sephadex G-50 spin columns (Amersham Biosciences). Tested and referenced DNA were mixed with 50 $\mu$g of human Cot-1 DNA (Invitrogen-Life Technologies, Inc.), precipitated, and resuspended in 45 $\mu$L of hybridization mixture consisting of 50% formamide, 10% dextran sulfate, 2× SSC, 4% SDS, and 10 $\mu$g/$\mu$L yeast tRNA (Invitrogen-Life Technologies, Inc.). The hybridization solution was heated to 73°C for 5 minutes to denature the DNA and then incubated for 45 minutes at 37°C to block repetitive sequences. The glass slides spotted with DNA were denatured in 70% formamide/2× SSC at 73°C for 4 minutes, then dehydrated in cold 70, 85, and 100% ethanol for 5 minutes each and air-dried. Hybridization was performed for 66 to 72 hours in a container on a slowly rocking table with 200 $\mu$L of 50% formamide/2× SSC, followed by posthybridization washings in 50% formamide/2× SSC for 15 minutes at 50°C, in 2× SSC/0.1% SDS for 30 minutes at 50°C, and in PN buffer (0.1 mol/L $NaH_2PO_4$ and 0.1 mol/L $Na_2HPO_4$ to attain pH 8 and 0.1% NP40) for 15 minutes at room temperature. The glass slides were then rinsed in 2× SSC at room temperature and finally dehydrated in 70, 85, and 100% ethanol at room temperature for 2 minutes each and air-dried. The slides were scanned with an Agilent Micro Array Scanner (Agilent Technologies, Palo Alto, CA), and the acquired array images were analyzed with Genepix Pro 4.1 (Axon Instruments, Inc., Foster City, CA). After automatic segmentation of the DNA spots and subtraction of the local background, intensities of the signals were determined. Subsequently, ratios of the signal intensity of two dyes (Cy3 intensity/Cy5 intensity) were calculated for each spot, converted into $\log_2$ ratios on an Excel sheet in the order of chromosomal positions, and then normalized. For centering the $\log_2$ ratio of normal components at 0, we normalized the $\log_2$ ratio of each of the samples according to the following method. The medium $\log_2$ ratio value for all clones was computed, and the clones were selected with a $\log_2$ ratio more than "the median + SD × A" or less than "the median − SD × A." "A" was visually defined as the normal region by referring to the $\log_2$ ratio plots of all clones for each experiment and was also assigned an approximate range from 0.5 to 1.0. We then computed the mean $\log_2$ ratio values for the selected clones and designated these value X. Finally, we obtained the Y value by subtracting X from the $\log_2$ ratio for each clone. In this study, each of the $\log_2$ ratios was analyzed on the basis of the Y value.

For the array, six simultaneous hybridizations of normal male *versus* normal male were performed to define the normal variation for the $\log_2$ ratio. A total of 122 clones with <10% of the mean fluorescence intensity of all of the clones, with the most extreme average test over reference ratio deviations from 1.0 and with the largest SD in this set of normal controls, was excluded from additional analyses. Thus, we analyzed a total of 1966 clones (covered 2988 Mb, 1.5 Mb of resolution) for additional analysis. A total of 1907 clones (covered 2834 Mb) of 1966 was from 1ptel to 22qtel. Because >96% of the measured fluorescence $\log_2$ ratio values of each spot (2 × 1966 clones) ranged from +0.2 to −0.2, the thresholds for the $\log_2$ ratio of gains and losses were set at the $\log_2$ ratio of +0.2 and −0.2, respectively. Linearity between copy number and values of $\log_2$ ratio was confirmed by the use of human fibroblast cell lines (22) that had different copy number of X chromosomes as described previously (10).

**Fluorescence *In situ* Hybridization Analysis.** Metaphase and interphase chromosomes were prepared from normal male lymphocytes and OCI LY13.2 cell line. Dual color fluorescence *in situ* hybridization analysis was conducted as described previously (10, 23).

**Statistical Analysis.** To analyze genomic regions for statistically significant differences between the two patient groups, the data set was constructed by defining genomic alterations as copy number gains for $\log_2$ ratio thresholds of +0.2 or more and as copy number loss for thresholds of −0.2 or less. Clones showing a gain ($\log_2$ ratio > +0.2) were inputted as 1 *versus* no-gain clones ($\log_2$ ratio ≤ +0.2) as 0 on an Excel template for each case. Similarly, loss clones ($\log_2$ ratio < −0.2) were inputted as 1 *versus* no-loss clones ($\log_2$ ratio ≥ −0.2) as 0 on another Excel template for each case. Cases showing genomic gain or loss were counted with Excel for each single clone (1966 clones in total) in the $CD5^+$ group or $CD5^-$ group. Data analyses were then carried out for the following purposes: (*a*) comparison of frequencies of gain or loss of each single clone between the $CD5^+$ and $CD5^-$ groups (1966 tests each for gain and loss, 3932 tests in total); (*b*) comparison of overall survival between cases showing gain or loss of a single clone and cases without respective gain or loss (1966 tests for each gain and loss with or without CD5 expression, in total 7864 tests maximum). Fisher's exact test for probability was used for the former comparison, and a log-rank test for comparing survival curves of the two groups was used for the latter. $P$ for screening of candidate clones for an analysis was <0.05. When a candidate clone was identified, the clone's continuity with the subsequent clones was examined. In cases where the $n$th clone and succeeding $k$ clones ($k \geq 0$) were found to be candidate clones, the $P$ value for continual association was calculated as:

$$\prod_{i=n}^{n+k} p_i$$

on the assumption that each clone is independent throughout the entire genome. Because multiple tests (11,796 tests maximum) were used, the conventional Bonferroni procedure was applied to define the $\alpha$-error for the final conclusion. Therefore, we defined a value for the calculation given above as $< 0.05/12{,}000 (= 4.2 \times 10^{-6})$ as statistically significant (24). All of the statistical analyses were conducted with a statistical package STATA, version 8 (College Station, TX).

## RESULTS

**Genomic Profiles and Data Analysis for DLBCL Cases.** Array CGH analysis was performed to compare genomic alterations in $CD5^+$ and $CD5^-$ DLBCL cases. All of the clones on chromosome X (57 clones) were separately analyzed because of sex mismatching. Of the 70 DLBCL cases enrolled, 4 cases (1 of $CD5^+$ DLBCL and 3 of $CD5^-$ DLBCL) did not show any genomic aberrations. The remaining 25 cases of $CD5^+$ DLBCL and 41 cases of $CD5^-$ DLBCL were then subjected to the data analysis. Fig. 1 shows the entire genomic profiles of two representative $CD5^+$ samples (Fig. 1, *A* and *B*) and one $CD5^-$ sample (Fig. 1C). Copy number changes were easily detectable at a high-resolution genome widely. Regions of high-level gain/amplification (defined as $\log_2$ ratio > +1.0), and regions suggestive of homozygous loss/deletion (defined as $\log_2$ ratio < −1.0) were also easily detected, as were regions showing low-level gain/amplification

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